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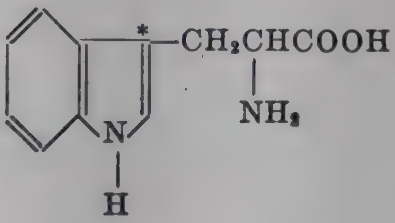
CORRECTIONS

On page 606, Table II, Dextran F, Vol. 176, No. 2, November, 1948, read *14.7* for $<$*14.7*.

On page 1461, Vol. 176, No. 3, December, 1948, the structure for tryptophan should read



THE EFFECT OF PARTIAL HEPATECTOMY, THERMAL INJURY, AND β-CHLOROETHYL VESICANTS ON THE LIPIDES OF PLASMA AND PLASMA FRACTIONS OF RATS*

By ALFRED CHANUTIN and ERLAND C. GJESSING

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(Received for publication, August 23, 1948)

A review of the literature indicates a paucity of information concerning any relationship between injury and lipide changes in the plasma of animals or man. After severe injury to dogs, the lipide concentration of several plasma protein fractions was increased, while that of the whole plasma was not altered (1). The present investigation was undertaken to examine in detail the lipides of plasma and plasma protein fractions of rats subjected to partial hepatectomy, to scalding, and to intravenous injection of a toxic agent (nitrogen mustard).

Methods

Inbred male rats of Wistar stock, about 70 days old and weighing between 150 and 200 gm., were used as experimental animals. In the first group, partial hepatectomy was performed according to the procedure of Higgins and Anderson (2); 60 to 75 per cent of total liver was removed; laparotomized animals served as controls. Rats in the second group were scalded by dipping the clipped backs in hot water at 75° for 40 seconds, under anesthesia, so that approximately one-third of the body surface was affected. In the third group, approximately 0.3 ml. of a freshly prepared saline solution of the nitrogen mustard, tris(β-chloroethyl)amine hydrochloride (HN3), was injected into the exposed jugular vein, so that the dose was 0.6 mg. per kilo; saline-injected rats were used as controls. The partially hepatectomized and scalded rats were allowed to have food (Purina checkers and Gaines dog meal) and water, while the animals injected with HN3 were fasted after injection. All experimental procedures were carried out under pentobarbital anesthesia. Blood was withdrawn from the abdominal aorta and was prevented from clotting with heparin.

In order to obtain a minimum of 20 ml. of plasma for fractionation, it was necessary to sacrifice a number of rats and pool the blood for any given experimental period. Four fractions of rat plasma were obtained according to the procedures described by Gjessing and Chanutin (3).

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* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Virginia. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

The fractions and plasma were dried in a desiccator over P_2O_5 *in vacuo;* they were crushed in a mortar and returned to the desiccator until the dry weights were constant. Weighed aliquots of the dried fractions were placed in extraction thimbles and were extracted on a steam bath while immersed in absolute alcohol for 5 hours; this was repeated with absolute alcohol-ethyl ether (1:1) for 5 hours. These two extracts were combined and brought to a total volume of 100 ml. with the alcohol-ether mixture. Aliquots of this extract were used for the determination of cholesterol by a modification of the Schoenheimer-Sperry (4) procedure. A 25 ml. aliquot was evaporated to dryness and the residue was dissolved in and the final volume brought to 25 ml. with petroleum ether. Aliquots of this extract were used for the determination of total lipide carbon (5) and for phospholipide phosphorus (6). Cholesterol and phospholipide values were converted to carbon by standard factors (1).

Results

Data for total lipide, cholesterol, and phospholipide carbon of plasma and plasma fractions of rats are presented in Figs. 1, 2, and 3 at varying intervals after partial hepatectomy, scalding, and injection of a toxic agent (HN3). Each point on the chart represents a pooled sample from a number of rats. In several instances the values represent the average of several determinations on different groups.

Partial Hepatectomy (*Fig. 1*)—Results obtained during the first 10 days after partial hepatectomy or laparotomy are shown. The lipide constituents of whole plasma are increased slightly. The pronounced variations of the total lipide concentrations of Fraction I of control and experimental animals are probably attributable to the trauma of the surgical procedure; the increase in phospholipide and cholesterol concentration on the 2nd day appears to be associated with liver insufficiency. During the first 4 days, the lipide values of Fraction II+III of the partially hepatectomized rats are elevated, while those of the controls show little or no change. The lipides of Fraction IV-4 of the laparotomized groups decrease immediately after operation, while the values of the partially hepatectomized animals remain fairly constant. The albumin-rich Fraction V contains traces of lipides whose concentrations are not appreciably affected.

Scalding (*Fig. 2*)—A small but definite increase in the lipide concentration of the plasma is observed after severe thermal injury. The total lipides of Fraction I decrease at the 12th hour and reach a minimum on the 3rd day; small decreases in the concentrations of phospholipide or choles-

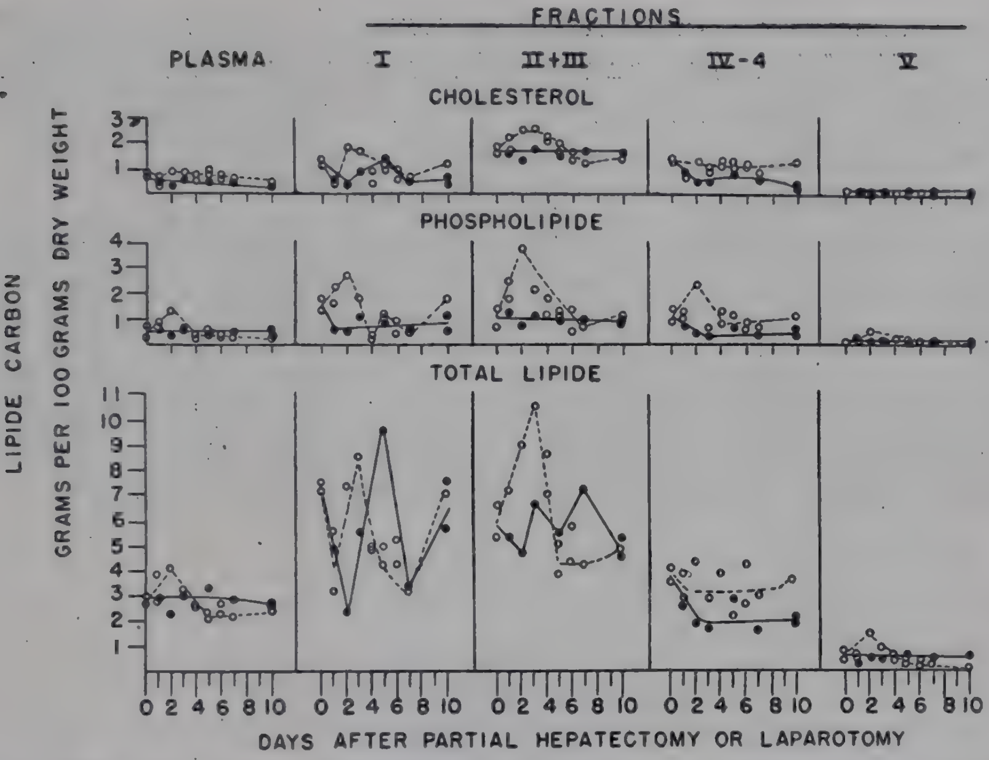

FIG. 1. Effect of partial hepatectomy on the lipides of plasma and plasma protein fractions. The dotted lines represent partially hepatectomized animals and the solid lines represent the laparotomized animals.

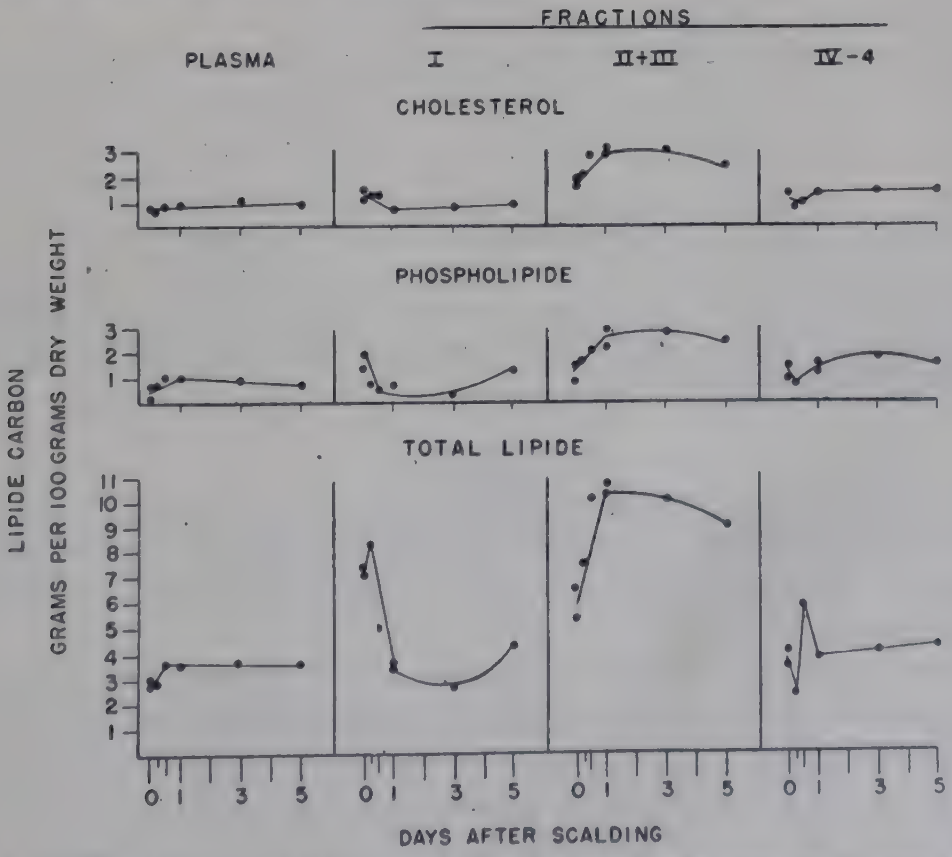

FIG. 2. Effect of thermal injury on the lipides of the plasma and plasma protein fractions.

terol are observed. An immediate pronounced increase in the lipides of Fraction II+III is seen, which reaches a maximum after 24 hours; the values gradually decrease but remain elevated on the 5th day. The daily variations in the concentration of lipides of Fraction IV-4 are small. No change in the concentration was seen in Fraction V.

Nitrogen Mustard (*HN3*) (*Fig. 3*)—The total lipide, phospholipide, and cholesterol concentrations of the HN3-injected animals are consistently elevated above those of the saline controls in the plasma and in Fraction IV-4. The total lipide concentrations of Fraction II+III are markedly elevated on the 3rd day after injection; the values for the saline groups decrease and remain low. The lipide carbon for Fractions I and V shows no appreciable change as a result of injection of a nitrogen mustard.

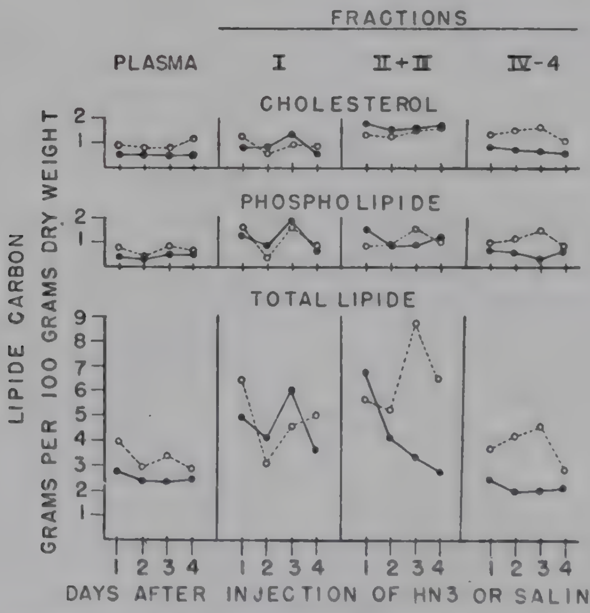

FIG. 3. Effect of intravenous injection of HN3 on the lipides of the plasma and plasma protein fractions. The dotted lines represent the HN3-injected and the solid lines the saline-injected animals.

DISCUSSION

The preceding data demonstrate that the concentrations of lipides, associated with the plasma proteins of Fraction II+III, are consistently increased at the time when metabolic processes are acutely affected by injury or by a toxic agent.

The concentrations of the components of the regenerating liver of partially hepatectomized rats may undergo marked changes. Liver growth is most rapid on the 2nd and 3rd days (2, 7), during which time the concentration of pentose nucleic acid is markedly increased (8). During the 1st day increased amounts of lipide are deposited in the liver remnant; on the 2nd and 3rd days the lipide concentration decreases rapidly (9). The data in these experiments indicate that the total lipide, phospholipide, and cholesterol, particularly in Fraction II+III, increase during the period of greatest liver regeneration.

After the rats were scalded, the proteins of Fraction II+III show marked changes in distribution within a few hours (3). A marked decrease in the cholesterol ester and ascorbic acid concentrations of the adrenals is seen shortly after thermal injury (10). Within a few hours after burning, the total lipides decrease markedly in Fraction I and remain depressed; in Fraction II+III they increased sharply and remained elevated. The phospholipide and cholesterol concentrations follow the trend of the total lipide. These changes are definitely associated with the severe acute thermal injury.

After the intravenous injection of an LD_{100} dose of HN3 into rats, leucopenia, enteritis, and diarrhea are first manifested prominently on the 3rd day. At this time, the adrenal cholesterol ester concentration is most depressed (10) and the fatty infiltration of the thymus nuclei is most pronounced (11). In the present experiments, the elevations of total lipide carbon concentrations of Fractions II+III and IV-4 are most marked on the 3rd day after injection.

SUMMARY

The concentrations of total lipide, phospholipide, and cholesterol carbon of the plasma and four plasma fractions of rats were determined after partial hepatectomy, laparotomy, scalding, and after the injection of tris(β-choloroethyl)amine (HN3) and saline.

The concentrations of the lipide constituents of whole plasma were slightly increased in the injured animals. The total lipide concentration of Fraction II+III was always elevated when the effects of these injuries were most pronounced. Changes in the lipide concentrations of the remaining fractions appeared to depend on the experimental procedures.

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THE SERINE REQUIREMENT OF STREPTOCOCCUS FAECALIS R AS A FUNCTION OF THE BASAL MEDIUM

BY BRYANT R. HOLLAND AND W. W. MEINKE

WITH THE TECHNICAL ASSISTANCE OF MELBA BREEDLOVE SAMPLE

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(Received for publication, September 3, 1948)

In a previous publication, in which the authors (1) reported an antagonism between serine and threonine, it was postulated that *Streptococcus faecalis* R might be suitable for a serine assay, if the effects of other factors on the serine requirement were more clearly understood. Baumgarten *et al.* (2) have reported the use of *Streptococcus faecalis* R for the microbiological assay of serine, and others (3, 4) have reported serine to be essential for this organism, while Dunn *et al.* (5) found serine to be non-essential.

Subsequent work in this laboratory indicates that the *Streptococcus faecalis* R requirement for serine is a function of the basal medium employed, rather than being due to unsuspected amino acid impurities. In addition to threonine (1), it was found that adenine, guanine, uracil, folic acid, citrate, and ferrous iron altered the requirement for serine.

EXPERIMENTAL

Source of Materials—The folic acid used in these experiments was synthetic pteroylglutamic acid (folvite) obtained from the Lederle Laboratories Division, American Cyanamid Company. The folic acid concentrate, potency 4000, was prepared from spinach and was obtained from Dr. R. J. Williams, Biochemical Institute, The University of Texas.

Methods—The experimental technique employed and the culture of *Streptococcus faecalis* R used were the same as those described in an earlier publication (1). Medium II of Baumgarten, Mather, and Stone (2), unless otherwise indicated, was used with modifications shown in the tables.

Results

A comparison of three different media (Table I) for *Streptococcus faecalis* R shows gross differences in the serine growth response. With Medium A of Baumgarten *et al.* (2), serine would be classed as an essential metabolite, while with Medium B of Lyman *et al.* (6) and Medium C of Stokes *et al.* (7) it would be classed as stimulatory.

Effect of Adenine, Guanine, and Uracil—It is evident (Table II) that these compounds inhibit growth in the absence of serine and in the presence of limiting serine. Individual and combined effects of adenine, guanine, uracil, and xanthine tested in the absence of serine (Fig. 1, Curve B) vary with the compound tested. With as little as 25 γ of DL-serine present, the toxicity is considerably diminished (Fig. 1, Curve C). The toxicity of these compounds decreases in the order adenine, guanine, uracil,

TABLE I

Response of Streptococcus faecalis R on Different Serine-Free Media

The figures in parentheses refer to the bibliography. No xanthine was added to any of the media. Pyridoxine, 16 γ and 20 γ respectively, was used in Media B and C, instead of pyridoxamine.

DL-Serine	Medium A (2)	Medium B (6)	Medium C (7)
	Titration, 0.1 N NaOH		
γ	*ml.*	*ml.*	*ml.*
0	0.0	6.43	2.50
4	0.35	6.56	3.01
10	1.90	6.90	3.44
20	4.01	6.63	3.75
30	5.61	6.84	3.80
40	5.92	6.97	3.70
60	6.33	7.33	3.22
80	6.90	7.63	3.17
120	7.41	8.24	3.25
160	7.87	8.70	3.62
200	7.37	8.90	4.15

TABLE II

Inhibitory Effect of Adenine, Guanine, and Uracil

Adenine, guanine, and uracil*	No serine	20 γ DL serine
	Titration, 0.1 N NaOH	
mg.	*ml.*	*ml.*
0.0	4.04	5.92
0.05	1.60	5.50
0.10	0.75	5.33
0.20	0.43	4.75
0.30	0.15	4.63
0.40	0.06	3.75
0.50	0.0	3.31
0.75	0.0	1.65
1.00	0.0	0.0

* The concentration represents the amount of each constituent present, with 0.05 γ of folic acid per 10 ml. tube.

with no toxicity shown by xanthine. These pronounced inhibitory effects of the purines and pyrimidine have been observed only in the absence of serine or with limiting serine. In Table III these effects are demonstrated for serine and are shown to be absent for threonine and valine. With threonine and valine the results are essentially independent of the adenine, guanine, and uracil concentration.

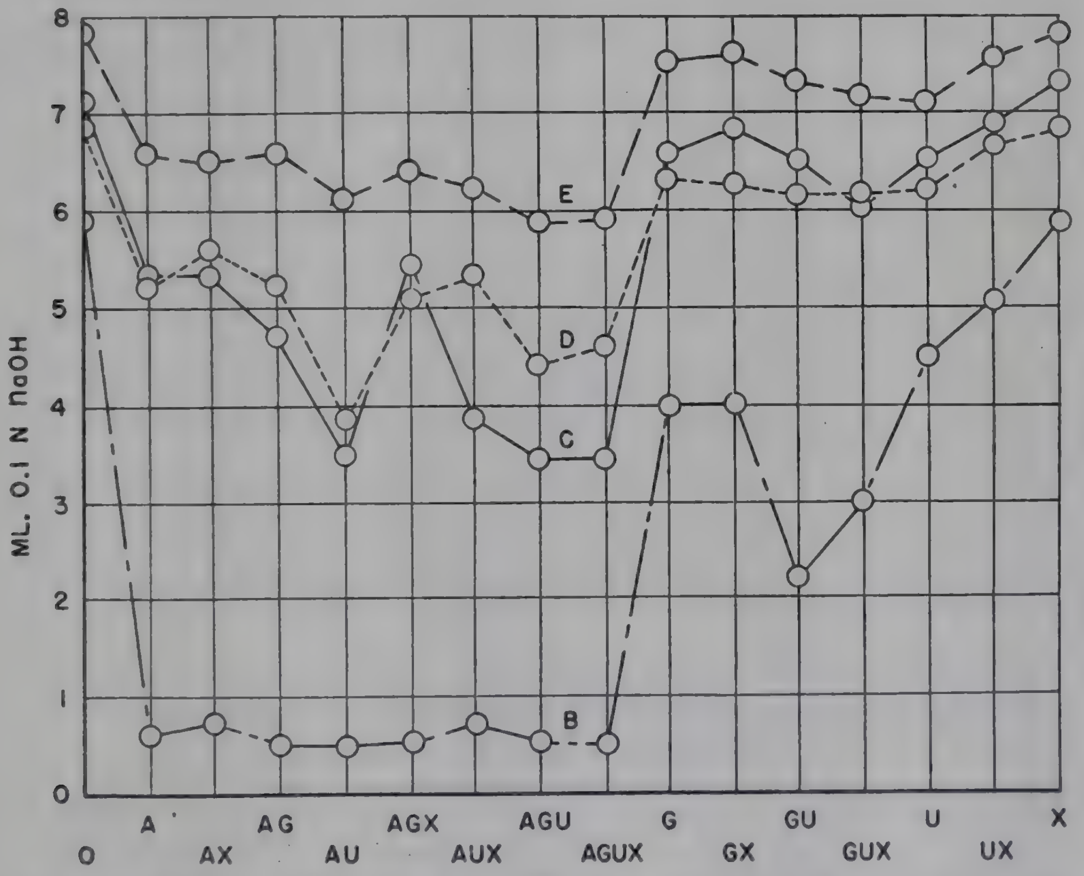

FIG. 1. The growth response of *Streptococcus faecalis* R to adenine (*A*), guanine (*G*), uracil (*U*), and xanthine (*X*) in the presence of the following: Curve B, no serine and 0.05 γ of folic acid; Curve C, 25 γ of DL-serine and 0.05 γ of folic acid; Curve D, no serine and 5 γ of folic acid; Curve E, 25 γ of DL-serine and 5 γ of folic acid. The abscissa represents 0.5 mg. each of *A*, *G*, *U*, and *X* as indicated.

In the absence of adenine, guanine, and uracil there is an appreciable change from the reported inhibition ratio of threonine to serine (1) of 2500. Such a change is evidenced by the titrations shown in the column for 0.05 γ of folic acid, Table IV. The titration of 1.32 ml. of 0.1 N NaOH was obtained under conditions previously reported; namely, 0.5 mg. each of adenine, guanine, and uracil and 0.05 γ of folic acid. Adenine alone has essentially the same inhibitory effect of the three combined but the addition of either guanine or uracil alone or complete absence of the purines and pyrimidine significantly increases the titration. These increases in titration clearly indicate that a ratio of threonine to serine of 2500 is no longer inhibitory.

Table III

Growth Response to Serine, Threonine, and Valine As Influenced by Adenine, Guanine, Uracil, and Folic Acid

Amino acid		Folic acid	Adenine, guanine, and uracil 0 mg.	0.1 mg. each	1.0 mg. each
			Titration, 0.1 N NaOH		
	mg.	γ	*ml.*	*ml.*	*ml.*
DL-Serine	0.0	0.05	5.00	2.58	0.78
	0.05	0.05	7.60	7.48	4.96
	0.0	5.0	6.48	5.40	1.88
	0.05	5.0	7.95	8.10	7.20
DL-Threonine	0.0	0.05	1.00	1.25	1.00
	0.05	0.05	5.30	5.65	5.10
	0.0	5.0	1.05	1.25	0.95
	0.05	5.0	4.92	5.80	5.60
DL-Valine	0.0	0.05	1.70	2.00	1.65
	0.05	0.05	4.94	5.46	5.16
	0.0	5.0	1.70	1.85	1.90
	0.05	5.0	4.65	5.40	5.35

Table IV

Effect of Adenine, Guanine, Uracil, and Folic Acid on Growth at Threonine-Serine Ratio of 2500

25 mg. of DL-threonine and 0.01 mg. of DL-serine in each tube.

Purine and pyrimidine added*	0.05 γ folic acid	5.0 γ folic acid
	Titration, 0.1 N NaOH	
	ml.	*ml.*
Adenine, guanine, uracil	1.32	3.74
Adenine	1.84	5.48
Guanine	4.85	6.44
Uracil	4.94	6.00
None	5.52	6.59

* 0.5 mg. of each as indicated per 10 ml. tube.

Effect of Folic Acid—Concentrations of folic acid of 0.05 γ per tube (6) and 0.1 γ per tube (8), which are satisfactory under most assay conditions, do not suffice with limiting serine. Greatest growth is obtained under serine blank conditions with 5 γ of folic acid (Table V). This effect is also shown in Table IV, where increased folic acid changes the inhibition ratio of threonine to serine, and in Table III and Fig. 1, Curve D, where increased folic acid overcomes the toxicity of the purines and uracil in the presence of limiting or no serine.

TABLE V

Toxicity Test for Folic Acid and Folic Acid Concentrate

Folic acid*	Titration, 0.1 N NaOH	Folic acid†	Titration, 0.1 N NaOH	Folic acid† concentrate	Titration, 0.1 N NaOH
γ	*ml.*	γ	*ml.*	γ	*ml.*
0.00	0.00	0.00	0.40	0	0.72
0.05	0.00	0.05	3.60	2	5.71
0.50	1.50	0.50	6.33	20	7.20
5.00	3.58	5.00	6.80	200	7.40
50.00	3.10	50.00	6.19	800	7.91
500.00	0.78	500.00	2.65	2,000	8.40
2500.00	0.49	2500.00	0.66	20,000	9.30

* 0.5 mg. each of adenine, guanine, and uracil present.

† 0.1 mg. each of adenine, guanine, and uracil present.

TABLE VI

Reversal of Folic Acid Toxicity by Serine and Folic Acid Concentrate

The adenine, guanine, and uracil concentration is 0.1 mg. of each per 10 ml. tube.

Folic acid	DL-Serine*		Folic acid concentrate†	
		Titration, 0.1 N NaOH		Titration, 0.1 N NaOH
γ	γ	*ml.*	γ	*ml.*
500	0	2.03	20	2.16
500	25	5.07	100	3.10
500	50	6.47	200	3.66
500	100	8.18	500	4.50
500	200	9.28	1000	5.41
5	0	5.40	20	6.66
5	25	6.75	100	6.71
5	50	7.61	200	6.85
5	100	8.70	500	7.49
5	200	9.60	1000	8.11

* No folic acid concentrate present.

† No serine present.

It is to be noted (Table V) that high levels of folic acid become inhibitory, while such is not the case with folic acid concentrate. Both serine and folic acid concentrate overcome the high folic acid inhibition (Table VI).

Effect of Citrate and Fe^{++}—Results in this laboratory have shown consistently that citrate inhibits growth under limiting serine conditions. This is illustrated by the first set of titrations in Table VII. The report of Henderson and Snell (8), indicating competition between Mn^{++} and citrate for some lactic acid bacteria. led to the experiments described in Table VIII. Under these conditions (no serine, 2 per cent citrate, 0.5 mg. of the purines and pyrimidine, and 5 γ of folic acid) it is noted that an increase in the level of Salts B[1] causes increased growth. As also noted from Table VIII, this response is due to Fe^{++}. On the addition of 500 γ of Fe^{++} (Table VII) the inhibitory effect of citrate is eliminated, and the typical response due to increased buffer capacity appears.

TABLE VII

Effect of Citrate and Fe^{++} on Serine Blank

0.5 mg. each of adenine, guanine, and uracil; 5 γ of folic acid per 10 ml. tube.

Citrate	20 γ* Fe^{++}	500 γ Fe^{++}
	Titration, 0.1 N NaOH	
mg.	*ml.*	*ml.*
0	3.68	3.40
25	3.50	3.80
50	3.20	4.25
100	2.62	4.64
200	2.62	5.10

* Quantity present as Salts B.

TABLE VIII

Effect of Fe^{++}, Mn^{++}, and Mg^{++} on Serine Blank

2 per cent citrate, 5 γ of folic acid, and 0.5 mg. each of adenine, guanine, and uracil present.

Salts B	Titration, 0.1 N NaOH	Fe^{++}	Mn^{++}	Mg^{++}	Titration, 0.1 N NaOH
ml.	*ml.*	γ	γ	γ	*ml.*
0.0	1.35	20	25	200	1.35
0.05	1.35	50	25	200	1.60
0.0625	1.55	100	25	200	2.00
0.125	2.30	500	25	200	4.45
0.3125	3.33	20	50	200	1.35
0.625	4.48	20	100	200	1.30
1.25	4.42	20	500	200	1.40
		20	25	500	1.32
		20	25	800	1.88
		20	25	1000	1.30

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[1] See (14).

DISCUSSION

Apparently serine is required by *Streptococcus faecalis* R for growth, although under certain conditions much of the needed serine may be synthesized. The various factors involved tend to complicate any explanation as yet conceived. The facts, however, indicate that folic acid is at least partially responsible for the serine synthesis. In no case has the need for excessive quantities of folic acid been demonstrated except under serine blank conditions. This is indicative of serine synthesis due to folic acid. An analogous situation might be the synthesis of lysine, threonine, and alanine by *Lactobacillus arabinosus* in the presence of pyridoxine (7, 9). These amino acids were required in the absence of pyridoxine.

Adenine, guanine, and uracil are usually incorporated in microbiological assay media. Snell and Mitchell (10) found *Streptococcus faecalis* to require adenine and thymine in the absence of folic acid. Stokes (11) concluded that good growth cannot be obtained in the absence of purine bases with either thymine or folic acid. However, Rabinowitz and Snell (12), in assaying for vitamin B_6, omitted adenine, guanine, and uracil from the medium because these substances never stimulated growth and were sometimes slightly inhibitory. Also, Henderson and Snell (8) have shown that the purines and pyrimidine bases, either omitted or added (up to 0.2 mg. per 10 ml. tube), had no effect on the leucine assay by *Streptococcus faecalis* R.

Under our conditions, somewhat different results were obtained. Adenine produced the most marked inhibition in tubes with little or no serine, and in no case did any of the purine and pyrimidine combinations, except xanthine alone, produce growth equivalent to the growth in their absence (Fig. 1). However, in the presence of adequate serine (Table III), the presence of 0.1 mg. each of adenine, guanine, and uracil was slightly stimulatory, while the 0.5 mg. level was slightly inhibitory. The incorporation of 0.5 mg. per tube of these compounds in the basal medium by Baumgarten *et al.* (2) represents a marked change from most media and is largely responsible for the serine blank obtainable with this medium.

It is not yet clear how adenine adversely affects the synthesis of serine. Folic acid partially overcomes the inhibition of adenine. The most plausible explanation the authors can give at present is that folic acid is a coenzyme for the synthesis of a specific compound (probably purine-like) which is necessary for the synthesis of serine. The adenine in the media would then become competitive with this "specific compound." Unpublished data from this laboratory indicate that this compound is not thymine or hypoxanthine.

The toxicity of the folic acid preparation, observed with limiting serine conditions, is counteracted by serine (Table VI). The reversal of the folic acid toxicity by folic acid concentrate (potency 4000) is believed to be due to the presence of serine in the preparation, as an acid hydrolysate of the concentrate was as active in overcoming the inhibition as was the concentrate.

The previously reported (1) threonine-serine ratio of 2500 which inhibited growth is merely a function of the basal medium. Any constituent which promotes the synthesis of serine would automatically increase the ratio, while any constituent which inhibits the synthesis of serine would decrease the ratio. These conditions do not alter the antagonism between serine and threonine; they merely change the amount of serine present.

The superiority of citrate as a buffer for *Streptococcus faecalis* R has been claimed by several workers (8, 13, 14). In a study of the mineral requirements (MacLeod and Snell (14)) and in the leucine assay (Henderson and Snell (8)) with *Streptococcus faecalis* R, citrate buffer did not increase the need for Salts B. Extra manganese and magnesium were required with other lactic acid bacteria, owing to formation of complexes between citrate and these bivalent ions. The situation is quite different with serine assay conditions (Tables VII and VIII) in that citrate does inhibit growth and manganese or magnesium has no effect. The fact that iron eliminates the citrate toxicity suggests that iron is involved in the biosynthesis of serine. In this case the effect of citrate is secondary and only appears in the presence of inadequate iron and serine.

Streptococcus faecalis R has been used in the microbiological assay for serine. As a result of present and previous findings, however, the method is subject to severe limitations, and the results obtained are of questionable accuracy, since under the proper conditions the organism is capable of synthesizing its serine requirement. A serine blank may be obtained by the use of a heavy dose of adenine. Under this condition folic acid is stimulatory. The incorporation of additional folic acid in the medium tends to destroy the blank, while excessive amounts of folic acid become toxic. Citrate may also be used to obtain a serine blank. This, however, is counteracted by the addition of iron. Finally, threonine is antagonistic to the utilization of serine. If the threonine content of the medium is increased sufficiently to eliminate any effects produced by the addition of threonine in the sample, a lag section appears in the standard curve which decreases the sensitivity of the assay. It is our opinion that *Streptococcus faecalis* R is not a satisfactory organism for the assay for serine.

SUMMARY

1. Folic acid has been shown to be effective in promoting the synthesis of serine by *Streptococcus faecalis* R.

2. Adenine and to a lesser extent guanine and uracil inhibit the synthesis of serine.

3. Citrate, under limiting serine conditions, is inhibitory to growth of *Streptococcus faecalis* R. This inhibition is reversed by ferrous iron.

4. The effect of various medium constituents on the serine assay was discussed.

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THE NITROGENOUS CONSTITUENTS OF THE TISSUE LIPIDES

I. THE EXTRACTION, PURIFICATION, AND HYDROLYSIS OF TISSUE LIPIDES*

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Data are available from a number of laboratories (1–4) on the content of lipide nitrogenous substances in isolated tissues of several species or in lipide fractions of tissues. Artom has described techniques for the determination of tissue lipide choline, total phospholipide and sphingomyelin (5), and lipide ethanolamine and serine (6). Artom and Fishman have also determined total phospholipide and lipide choline in an extensive study of the composition of rat liver lipides in certain nutritional states (7–12). In this and in subsequent reports we wish to describe a study of the nitrogen base pattern of dog tissue lipides under a variety of experimental conditions.

In order to determine total lipide nitrogen and some individual bases it is necessary to free the lipide extract of its nitrogenous impurities. Page *et al.* (13), Van Slyke *et al.* (14), Folch and Van Slyke (15), and Christensen (16) have found heavy contamination of plasma lipide extracts with non-lipide nitrogen, principally urea. Swingle and Pfiffner (17) found adrenalin present in adrenal lipide extracts. Evidently a variety of nitrogenous substances ordinarily insoluble in lipide solvents are soluble in solutions of phosphatides. Many investigators have recognized this contamination, but little has been reported in the direction of (*a*) quantitative removal of impurities, or (*b*) measurement of the loss of lipides incurred in the purification procedure. Folch and Van Slyke (18) have reported a method of precipitation of plasma lipides and proteins which minimizes contamination with nitrogenous impurities. The losses of the lipides were not quantitatively described. Sinclair (19) purified emulsions of plasma phosphatides by dialysis against water. Losses of weight, nitrogen, phosphorus, and choline were determined, but the actual amounts of lipide and impurities lost are uncertain. We have not used precipitation techniques because of the danger of incomplete precipitation of the variety of lipides containing nitrogen. We have not used dialysis because it is not certain that all impurities in lipide extracts are dialyzable and because there is no as-

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surance that lipide losses might not occur in this process. We have purified lipide extracts in chloroform with minimum lipide loss by extraction in an emulsified state with 0.25 M $MgCl_2$. The lipide lost was measured by analyzing the $MgCl_2$ extracts for fatty acid and phosphorus.

We have determined total lipide nitrogen and phosphorus on these purified extracts of several dog tissues. In addition we have studied the conditions of hydrolysis of the lipides for the quantitative determination of choline.

EXPERIMENTAL

Preparation of Lipide Extracts—The animals were sacrificed by severing the carotid artery under deep magnesium or ether anesthesia. The tissues were quickly removed; a portion of 20 to 40 gm. was cut into conveniently sized pieces for grinding and dropped on dry ice in a mortar. The frozen tissue was then ground with dry ice, and the frozen powder added to alcohol-ether 3:1 in a ratio of roughly 40 ml. per gm. of fresh weight of tissue. By this procedure it was hoped to minimize artifacts produced by postmortem enzymatic hydrolysis of the tissue lipides described by Fairbairn (20). The ground tissue in alcohol-ether was then refluxed for 1½ hours, and filtered through sintered glassware. All of the tissue residue was transferred to the filter by rinsing the extraction flask with small portions of alcohol-ether. The residue was then dried in an oven at 50° and extracted in a Soxhlet extractor with $CHCl_3$ for 6 hours. Fatty acid analyses of the saponified residues by the oxidation method of Bloor (21) showed that only insignificant amounts of lipides remained in the residue. If the amount of unextracted fatty acid is calculated as monoaminophosphatide, the unextracted lipides represented from 0.23 to 0.63 per cent of the total amounts extracted.

The combined alcohol-ether and chloroform extracts were concentrated under reduced pressure in a stream of nitrogen. The residues were dissolved in chloroform and made to a convenient volume, usually 100 ml.

Purification of Lipide Extracts—Aliquots of the chloroform extract containing 0.4 to 2.0 mM of lipide nitrogen were placed in 250 ml. centrifuge bottles and diluted to about 80 ml. with chloroform. 80 ml. of 0.25 M $MgCl_2$ were added and the bottles were stoppered and shaken until relatively stable emulsions were produced. After standing for several hours at room temperature the emulsions were broken by freezing in a dry ice chamber, thawing, and centrifuging. The clear supernatant was removed by a pipette. Usually additional freezing, thawing, and centrifuging processes were required to break the emulsion completely. The whole process was repeated six or seven times for routine purification of the extracts. The purified lipide extracts in chloroform solution were transferred quantitatively to 200 ml. volumetric flasks. Aliquots from this solution were then used for analysis of total nitrogen, total phosphorus, and choline.

The efficiency of this procedure in removing impurities and in minimizing loss of lipides to the aqueous phase was studied by analysis of groups of pooled $MgCl_2$ extracts. Total nitrogen was determined by a micro-Kjeldahl procedure with selenium as catalyst and determination of ammonia in the distillate by nesslerization. Total phosphorus was determined by a superoxol-sulfuric acid digestion and analysis of the digest for inorganic phosphorus by the Fiske and Subbarow method. Fatty acids were determined by saponification of the concentrated washings with NaOH, acidification, and extraction of the free fatty acids with chloroform. The acid-water-washed chloroform extracts were then used for determination of fatty acids by the procedure of Bloor (21). Oxidizable material was referred to a standard determination by use of Eastman palmitic acid. This procedure was found to give the theoretical recovery of fatty acids from solutions of water-soluble soy bean phosphatides in similar concentrations. The results of these analyses are summarized in Table I.

It is apparent that the bulk of contaminant nitrogen is removed in the first three extractions with 0.25 M $MgCl_2$. However, significant amounts do remain and should be removed by further extractions. On the basis of the results given in Table I, six to nine extractions would be adequate for routine purification. Further extractions bring out nitrogen equivalent to only 1 or 2 per cent of the lipide nitrogen present. In the case of those tissue lipides which are more difficult to emulsify, *e.g.* intestine and skeletal muscle, several more extractions may be necessary.

The lipide losses incurred in the purification procedure can be calculated if the total fatty acids lost in the nine to twelve extractions are assumed to arise exclusively from monoaminophosphatide and the total lipide phosphorus in the purified extract is all assumed to be from monoaminophosphatide (Table I). It can be seen that the maximum phospholipide losses range from 0.92 to 3.64 per cent in the several tissues, a range which we believe is sufficiently low to be satisfactory. If the total fatty acids lost represent in part plasmal fatty aldehyde, the above lipide losses would be increased, depending upon the relative amounts of plasmal and monoaminophosphatide lost. Even if all of the lipide material were plasmal, the above figures would only be doubled, a magnitude of loss which still might not be considered prohibitive. Actually the phosphorus content of a number of the extracts is too low with respect to fatty acid or oxidizable material to be entirely plasmal phosphorus.

In assessing the loss of lipide material it should be emphasized that the measurement of the lipide substances in the extraction medium, as we have

TABLE I

Summary of Lipide Purification with 0.25 M $MgCl_2$

Tissue	Dry lipide-free residue	Total P in unpurified lipide extract	Total N in unpurified lipide extract	Molar ratio, N:P	Extractions 1–3			Extractions 4–6			Extractions 7–9			Extractions 10–12			Total P in purified lipide extract	Total N in purified lipide extract	Molar ratio, N:P	Lipide lost in $MgCl_2$ extracts as monoaminophosphatides	Minimum non-lipide P in original unpurified lipide extract
					Total P	Total fatty acids	Total N	Total P	Total fatty acids	Total N	Total P	Total fatty acids	Total N	Total P	Total fatty acids	Total N					
	gm.	*μM*	*μM*		*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*	*μM*		*per cent of total*	*per cent of total P*
Cerebrum	1.786	1372	3039	2.22	40.6	21.4	1108	16.9	20.9	100	14.8	20.8	60				1268	1801	1.42	2.49	1.40
" *	1.138	872	1946	2.23	27.4	5.39	722	16.7	8.82		12.2	9.48	24.9				795	1133	1.43	1.49	3.74
Skeletal muscle	7.521	528	1515	2.87	12.8	6.23	992	6.05	5.1	99	4.8	5.83	42	4.72	3.59	39	482	506	1.05	2.15	1.64
Pancreas	2.409	565	943	1.67	42.6	3.88	413	8.75	3.2	25	7.3	2.44	14.5				485	509	1.05	0.98	8.70
Kidney	3.109	611	1929	3.16	36.7	10.3	1153	8.85	5.1	39	6.7	5.25	21				542	642	1.18	1.91	5.17
" *	1.999	469	1925	4.10	39.6	4.63	1372	14.9	6.25		13.2	6.52	22.4				409	417	1.02	2.12	10.7
Heart	3.924	624	1893	3.03	27.9	4.24	1188	8.0	3.9	73	6.1	2.71	17				590	589	1.00	0.92	4.99
Intestine	4.728	553	1345	2.43	26.2	6.84	746	8.2	6.0	59	7.6	4.0	26	4.85	8.28	20	516	598	1.16	2.43	4.55
Liver*	2.952	527	950	1.80	43.1	5.74	552	23.2	8.75	63.4	7.00	5.15	14.4				478	454	0.95	2.05	10.2
Lung*	2.109	386	797	2.06	21.7	6.0	494	9.2	9.68		5.79	9.23	13.5				342	421	1.23	3.64	4.06

* Tissues from another animal. Purified by ten Extractions 1 to 4, 5 to 7, and 8 to 10 inclusive.

done, is more precise than measurement of the change in the composition of the lipide extract itself.

The amount of non-lipide nitrogen remaining in the purified extracts is not known. The extractable nitrogen has been decreased to a negligible amount, but impurities may remain which are not extractable with 0.25 M $MgCl_2$. The ratios of nitrogen to phosphorus in the washed lipide extract conform roughly to the expected values calculated from the estimated amounts of monoaminophosphatide and sphingolipides (22). We are, therefore, assuming as a working hypothesis that lipide extracts prepared in this manner are essentially free of nitrogenous impurities.

When water alone was used as a washing medium the loss of lipides was prohibitive. The fatty acid in the washings being calculated as monoaminophosphatide, the losses for liver were 3.9, brain 26.5, kidney 8.6, muscle 14.4 per cent for six washings, and heart 6.2 per cent after seven washings. We also attempted to purify the extracts with aqueous acetone solutions. Due to the high solubility of acetone in chloroform it was necessary to dissolve the lipides in petroleum ether. In an experiment with liver lipides washed only twice with (*a*) water, (*b*) 0.058 mole fraction of acetone in water, and (*c*) 0.125 mole fraction of acetone, the total lipide losses based on fatty acid-containing substances in the washings amounted to 3.35, 14.4, and 2.88 per cent, respectively, calculated as monoaminophosphatide. These losses, again, were considered prohibitive. The superiority of $MgCl_2$ solution to these other washing media in minimizing lipide losses may be explained as a "salting-out" effect or due to the formation of water-insoluble complexes with several of the lipides. Magnesium sulfate solutions were used by Folch and Van Slyke (18) as a washing solution.

Determination of Total Lipide Nitrogen—Total lipide nitrogen was determined by the Kjeldahl digestion with the digestion mixture recommended by the Association of Official Agricultural Chemists (23) for the macro-Kjeldahl method and with selenium as catalyst. For 100 ml. Kjeldahl flasks, 5 ml. of concentrated H_2SO_4, 2.5 gm. of anhydrous Na_2SO_4, and 0.1 gm. of $CuSO_4 \cdot 5H_2O$ were added conveniently as concentrated aqueous solutions. When the water was boiled off, an aliquot of the chloroform solution of the lipides sufficient to give 0.3 to 1.0 mg. of nitrogen was added along with 30 to 40 mg. of powdered selenium. More selenium was added after clearing, and the digestion was continued for 2 hours. The Kjeldahl digests were distilled from alkali into 8 ml. of 0.1 N HCl, and the distillate transferred to 50 ml. volumetric flasks. Suitable aliquots of this solution were nesslerized by the addition of 3 ml. of Nessler's reagent and water to a volume of 25 ml. The color was read in a Coleman universal spectrophotometer at 490 mμ. The range of 40 to 60 γ of nitrogen gave transmission readings of 64 to 53 per cent, respectively, which has been most suitable for our use.

This procedure is effective in the quantitative conversion of choline nitrogen to ammonia, whereas we have observed that several of the rapid micro digestion procedures are not. The inclusion of selenium is desirable because it contributes notable catalytic effects in this conversion. Erratic and low conversion has been experienced with the Folin-Denis (24) digestion procedure with sulfuric and phosphoric acids, the Koch-McMeekin (25) with sulfuric acid and hydrogen peroxide, and the Van Slyke (26) with sulfuric acid and potassium persulfate.

Determination of Total Lipide Phosphorus—Total phosphorus was determined on an aliquot of the purified lipide extracts containing 15 to 20 γ of phosphorus. The digestion procedure was essentially that of Youngberg and Youngberg (27) with use of 0.6 ml. of 1:1 H_2SO_4 with usually a total of 5 drops of Merck's reagent superoxol and a total digestion period of 3 minutes after clearing. After dilution and boiling to decompose pyrophosphate, 1 ml. of 10 per cent NaOH was added and the color developed with the reagents of Fiske and Subbarow (28) to a color volume of 10 ml. and read in the Coleman spectrophotometer at 660 mμ.

Hydrolysis—Suitable aliquots of the purified lipides in chloroform were placed in 200 ml., standard taper, round bottom flasks, and the solvent removed under reduced pressure and 65 ml. of saturated barium hydroxide added. A few glass beads were placed in the flask and the solution refluxed on a sand bath for 5 hours. With cerebrum lipides, however, a 7 hour hydrolysis was used. 6.5 ml. of concentrated HCl were added and the refluxing continued for $1\frac{2}{3}$ hours. The fatty acids and sphingosine were removed by chloroform extraction (22) and the hydrolysate analyzed for choline essentially by the procedure described by Handler (29).

The quantitative determination of several of the nitrogenous bases of the tissue lipides must depend upon the quantitative hydrolysis of the lipide. Various procedures for hydrolysis of the lipides have been reported. Thannhauser and coworkers (30) refluxed the lipides for 3 hours with a solution of gaseous HCl in methanol and stated that this was the only reagent used in their series which would give quantitative hydrolysis of sphingomyelin. Artom (5) also refluxed the lipides with 6 N HCl in methanol for 3 hours. Chargaff, Ziff, and Rittenberg (1) used 2 N aqueous H_2SO_4 for 40 to 48 hours in the quantitative hydrolysis of brain and heart phosphatides. Williams and his associates (31, 32) used a short hydrolysis in aqueous alcohol medium with $Ba(OH)_2$. Entenman *et al.* (33) heated the lipides in saturated $Ba(OH)_2$ over a steam bath for about 2 hours. Sperry and Brand (34) used saturated $Ba(OH)_2$ with a heating period of 2 hours for the determination of choline in brain lipides. In their experiments acid hydrolysis procedures were not satisfactory.

The liberation of inorganic phosphorus would be ideal as a criterion for the completeness of hydrolysis, since all of the nitrogenous substances are esterified to phosphorus except sphingosine in the glycolipides. The remarkable stability of α- and β-glycerophosphoric acid (35) and phosphorylcholine (36) to acid hydrolysis makes complete recovery of lipide phosphorus as inorganic phosphorus practically unattainable. We have found that inorganic phosphorus values in these hydrolysis procedures may be only one-third of digestion phosphorus values when choline liberation is maximum. With barium hydroxide hydrolyses the liberation of inorganic phosphorus is difficult to measure, probably because of absorption losses on barium precipitates. We have used the maximum liberation of choline as the criterion of hydrolysis as have other investigators (30–34), since choline appears to be completely stable to a 7 hour sand bath refluxing with saturated barium hydroxide.

In our experience aqueous H_2SO_4 and HCl methanol gave erratic and incomplete hydrolysis, when short heating periods were used. Prolonged refluxing (48 hours) with 2 N or 4 N H_2SO_4 gave quantitative results, but we considered it desirable to shorten the period of hydrolysis. Refluxing the lipides with saturated barium hydroxide on a sand bath for 4 hours appeared to give maximum liberation of choline in all of the tissue extracts except cerebrum, in which case we occasionally obtained low results. Routinely we have used a 5 hour hydrolysis period for all tissue extracts except cerebrum and a 7 hour period for cerebrum. These hydrolysates give maximum values for choline and good recovery of added choline.

Analysis of Dog Tissues—By the use of the foregoing analytical procedures the total lipide nitrogen, phosphorus, and choline in tissues of several normal adult dogs subsisting on stock ration have been measured and are given in Table II.

DISCUSSION

It may be noted from the data on the purification experiments that the first extractions with $MgCl_2$ contain considerably more phosphorus than do the later extractions (Table I). These amounts of phosphorus are considerably greater than the maximum possible amounts of phospholipide in the $MgCl_2$ extract calculated on the basis of fatty acid found. This indicates the presence of measurable non-lipide phosphorus impurity in the lipide extracts. The minimum amount of non-lipide phosphorus present in the washings can be calculated by assuming that all the oxidizable lipide material present is plasmal fatty aldehyde (one fatty aldehyde per phosphorus). This minimum per cent of impurity phosphorus in the original extracts shown in Table I varies from 1.40 to 10.7 per cent of the total phosphorus in the original lipide extract. If the fatty acids in the washings were present as monoaminophosphatide (two fatty acids per

TABLE II

Nitrogen, Phosphorus, and Choline Content of Dog Tissue Lipides

Tissue	Total lipide N per gm. dry lipide-free residue			Total lipide P per gm. dry lipide-free residue			Molar ratio, N:P			Total lipide choline per gm. dry lipide-free residue			Choline N, per cent of total lipide N		
	Dog 4	Dog 5	Dog 6	Dog 4	Dog 5	Dog 6	Dog 4	Dog 5	Dog 6	Dog 4	Dog 5	Dog 6	Dog 4	Dog 5	Dog 6
	μM	μM	μM	μM	μM	μM				μM	μM	μM			
Cerebrum	996	1008	1208	699	710	767	1.43	1.42	1.58	263	264	281	26.4	26.2	23.2
Skeletal muscle	73.8	67.3	72.9	74.7	64.1	70.7	0.99	1.05	1.03		33.8	32.7		50.1	44.9
Pancreas	178	211	184	180	201	175	0.99	1.05	1.05	108	120	97.5	60.6	57.0	53.1
Kidney	209	207	240	205	174	222	1.02	1.18	1.08	93.7	84.3	102.4	44.9	40.8	42.7
Heart	119	150	142	136	150	149	0.87	1.00	0.96		72.8	66.5		48.5	46.8
Intestine	125	127	139	108	109	115	1.15	1.16	1.21		55.5	57.5		43.9	41.3
Liver	154		114	162		108	0.95		1.05	91.0		56.8	59.1		49.8
Spleen	150		144	115		110	1.31		1.31	56.0		57.5	37.3		39.8
Lung	200		177	162		141	1.23		1.25	93.8		80.9	47.0		45.8

phosphorus), this minimum phosphorus impurity would be doubled. The impurity phosphorus present in the extracts of the same tissues from different animals varies considerably. Thus, the common procedure of calculating phospholipide concentrations from a determination of total phosphorus in crude lipide extracts might not be valid for some extracts. Contamination of lipide extracts with inorganic phosphorus is even a more troublesome factor in phospholipide turnover studies with radioactive inorganic phosphorus (37, 38).

Expression of concentration of the various lipide bases in terms of micromoles per gm. of dry lipide-free residue has been taken as a course of accuracy, convenience, and for a more significant method of expressing results. It greatly expedites the quantitative manipulation of frozen fresh tissue powder, since only the tissue which is extracted contributes lipide material and is weighed as the residue. It is essentially an expression of moles of lipide base per gm. of cellular metabolic substance and automatically rules out artifacts resulting from changes in dry weight or neutral fat in a tissue.

TABLE III

Average Lipide Phosphorus and Choline Content of Several Tissues of Rats (4), Dogs, and Cattle (2)†*

Tissue	Lipide P per gm. dry lipide-free residue			Lipide choline per gm. dry lipide-free residue		
	Artom (4)	This study	Kaucher *et al.* (2)	Artom (4)	This study	Kaucher *et al.* (2)
	μM	μM	μM	μM	μM	μM
Skeletal muscle	60	69.8	51.8	28.6	33.3	32.6
Heart	98	145	161	28.0	69.7	73.5
Brain	590	725	746	211	269	340
Liver	153	135	289	91.8	73.9	171
Kidney	131	200	171	69.6	93.5	121
Spleen	63	113		24.0	56.8	
Lung	90	152	158	54.2	87.4	94.5
Intestine		111	108		56.5	78.6

* Calculated from assumed percentages of moist tissue represented by the dry lipide-free residue.

† Calculated directly from the data given.

The results given in Table II show some rather surprising variations in the absolute amounts of lipide N, P, and choline in the tissues of the three dogs, particularly in liver, kidney, pancreas, and heart, and to a less extent the cerebrum. Whether or not this variation will occur in litter mates under controlled experimental conditions remains to be seen. The above animals were selected from stock at random and thus a variety of factors might contribute to these differences. Although the number of animals is extremely limited, the data suggest that the variation is greater in the absolute quantity of N, P, and choline per gm. of dry lipide-free residue than in the relation of these substances to each other. The N:P ratios and the per cent of total N represented by choline seem to be a more constant quantity from animal to animal. It may be that the pattern of these lipides is fairly constant and that variation may arise in the actual amount of this mixture laid down in the tissue.

The actual amounts of lipide phosphorus and choline present in the several tissues may be compared with the amounts calculated from the data of Kaucher *et al.* (2) in beef tissues. The data of Artom (4) from rat tissues cannot be used without assuming the per cent of moist tissue represented by the dry lipide-free residue. However, a rough comparison of the average values can be made and the data are summarized in Table III. The variation in the values for most of the tissues is considerable and suggests the possibility of important species and individual variations as well as differences resulting from the analytical methods used.

SUMMARY

The preparation and purification of lipide extracts of dog tissues is described. The extracts in chloroform solution are freed of non-lipide impurities by extraction in an emulsified state with 0.25 M $MgCl_2$. After six or seven such extractions the amount of nitrogenous material extracted becomes negligible. In unpurified extracts the non-lipide nitrogen is always considerable and may be several times greater than the lipide nitrogen. The non-lipide phosphorus is present in much smaller quantities and amounted to 1.4 to 10.7 per cent of lipide phosphorus in the extracts analyzed. The maximum total losses of lipides incurred in the purification as measured by fatty acid or oxidizable material passing into the aqueous phase is 0.9 to 3.6 per cent of the total calculated as monoaminophosphatide. The purification technique is, therefore, considered satisfactory for the nine tissues reported.

A technique for the determination of total lipide nitrogen is described. The amounts of nitrogen, phosphorus, and choline present in lipide extracts of nine tissues from several dogs are presented.

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THE NITROGENOUS CONSTITUENTS OF THE TISSUE LIPIDES

II. THE DETERMINATION OF SPHINGOSINE IN TISSUE LIPIDE EXTRACTS*

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Our projected studies on the quantitative distribution of the various nitrogenous bases in tissue lipides necessitated a method for the quantitative determination of sphingosine. Since we have been unable to find a single report in the literature on this problem, we have undertaken the development of such a method. It is the purpose of this paper to describe a relatively simple procedure for an approximate determination of sphingosine, together with some preliminary results of the application of the method to lipide extracts of dog tissues.

Sphingosine, 1,3-dihydroxy-2-amino-4,5-octadecene, and dihydrosphingosine (1), 1,3-dihydroxy-2-aminooctadecane, occur in tissue lipides as fatty acid amides and their derivatives. The general classes of compounds include the glycosides or cerebrosides, the sphingomyelins, and possibly others. Since these substances probably represent a considerable number of chemical individuals in any tissue, and since they have quite different solubility properties, we have made no attempt to separate the parent substances from the non-sphingosine-containing lipides of the tissue lipide extract, but have sought to determine total sphingosine after hydrolysis of all of the lipides present. The method to be described consists essentially of a separation of sphingosine from the other bases present in the hydrolysate by $CHCl_3$ extraction, followed by the determination of sphingosine in the extract as nitrogen.

Procedure

The lipides were extracted, purified, and then hydrolyzed with 65 ml. of saturated barium hydroxide solution as described previously (2), and 6.5 ml. of concentrated HCl were added and the refluxing was continued for $1\frac{2}{3}$ hours. The splitting of the glycoside linkage from the cerebroside products would thus be assured. The contents of the hydrolysis flask were then transferred to a 125 ml. separatory funnel with about 10 ml. of $CHCl_3$. The flask was dried in an oven at 60° overnight and the dry flask

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and reflux condenser were rinsed first with glass-distilled reagent $CHCl_3$, then with warm water. The rinsings were added to the separatory funnel. The volumes of the aqueous and $CHCl_3$ phases were about 75 and 20 to 25 ml., respectively. The extraction of sphingosine was accomplished by vigorous shaking, and the $CHCl_3$ layer was then withdrawn into a 50 ml. centrifuge tube; 10 to 15 ml. more $CHCl_3$ were added to the separatory funnel, and the extraction repeated. No emulsions were produced under these conditions although troublesome emulsions may be encountered with other solvents. The combined $CHCl_3$ extracts were cleared of a faint cloudiness (perhaps due to traces of $BaCO_3$ or water) by centrifuging. The clear chloroform solution was withdrawn by pipette into a 50 ml. volumetric flask. A third extraction of the aqueous phase was made with 10 to 15 ml. of chloroform, and this extract was centrifuged and transferred to the volumetric flask; chloroform was finally added to the 50 ml. mark. Suitable aliquots were then evaporated in 20 mm. Pyrex test-tubes containing 0.7 ml. of 1:1 H_2SO_4 and a glass bead, and the digestion for nitrogen carried out according to the method of Koch and McMeekin (3). The digestion was usually carried out with 10 to 12 drops of Merck's reagent superoxol. Care was taken to add the superoxol at the immediate appearance of charring and fumes as recommended by Miller and Miller (4). After most of the carbonaceous material was oxidized, the boiling of the acid was maintained at a vigorous rate to insure rinsing down and digestion of fatty acid deposited along the length of the tube. After digestion, the acid was diluted with 7 to 10 ml. of distilled water, cooled, and neutralized with 7 ml. of 1.0 N NaOH. The mixture was transferred to a graduated 25 ml. tube and 2 drops of gum ghatti and 3 ml. of Nessler's solution were added. The solution was then centrifuged for 5 minutes at 2000 R.P.M. to remove the very faint cloudiness due probably to traces of $BaSO_4$.

The nitrogen content was then determined by reading absorption in the Coleman spectrophotometer at 490 mμ against a water blank and assessing nitrogen from a standard curve. Nitrogen analyses of each extract were performed in triplicate.

EXPERIMENTAL

The specificity of the extraction procedure was tested by adding known amounts of ethanolamine, choline, and serine to a synthetic hydrolysis mixture containing pure stearic acid and triacetyl sphingosine. Triacetylsphingosine was prepared from crude beef brain cerebrosides, the glacial acetic acid precipitate fraction described by Levene (5), by the procedure of Carter and associates (1).

It can be seen from Table I that the extraction is quantitative and completely specific for sphingosine in the presence of the other bases known to be present in tissue lipide hydrolysates. It is interesting that quantitative extraction of sphingosine with chloroform can be obtained from a solution essentially 0.5 N with respect to HCl, at which sphingosine should exist as an ion. This extraction is also specific and quantitative at high pH ranges in which sphingosine would carry no charge.

TABLE I

Recovery of Sphingosine from Hydrolysis Mixtures

No. of samples	Hydrolysis mixture		Total sphingosine in hydrolysate extract	Sphingosine recovered	
	Lipides*	Triacetylsphingosine added	Average	Average	Range
		μM	*μM*	*per cent*	*per cent*
3	None	9.07	9.24	100.4	100.0–103.3
2	51 mg. stearic acid	8.98	8.62	96.1	93.1– 99.0
3	60.5 μM ethanolamine, 74.9 μM choline, 30.0 μM serine, 51 mg. stearic acid	8.98	8.61	95.9	92.4– 98.5
4	4 ml. cerebrum lipides		11.69		
1	5 “ “ “		15.38		
3	4 “ “ “	8.98	20.29	94.5	91.3–101.0
2	15 ml. kidney lipides		9.21		
2	12 “ “ “	9.07	15.11	85.5	84.6– 86.4
1	12 “ “ “	8.98	16.04	96.8	
2	15 “ intestine lipides		7.66		
1	15 “ “ “	9.07	15.51	86.6	
1	12 “ “ “	9.07	14.64	93.8	
1	25 “ heart lipides		9.79		
2	20 “ “ “		7.71		
2	20 “ “ “	9.07	16.64	98.1	95.8–100.4
3	25 “ skeletal muscle lipides		7.71		
1	25 ml. skeletal muscle lipides	9.07	16.53	98.0	

* 1 ml. of the lipide extracts is equivalent to the following quantities of dry lipide-free tissue: cerebrum 8.92 mg.; kidney 15.54 mg.; intestine 23.6 mg.; heart 19.6 mg., and skeletal muscle 27.6 mg.

Data from the determination of sphingosine in purified tissue lipide extracts and recovery experiments with triacetylsphingosine added to these extracts are also given in Table I. The recovery of sphingosine ranged from 84.6 to 101.0 per cent and averaged 93.0 per cent. Although this is lower than is desirable for the determination to be considered absolutely "quantitative" in the usual sense of the word, the method may be considered to give approximate values for sphingosine. Sphingosine, like choline, appears to be stable to the whole hydrolysis procedure. Some variations in the volume of $Ba(OH)_2$ used, the time of acid hydrolysis, and the total volume of $CHCl_3$ used in the extraction of sphingosine have been employed. The total $CHCl_3$ volume does not appear to be critical within the range of 25 to 50 ml. The large volume of $Ba(OH)_2$ used routinely is necessary for those tissues containing little sphingosine and for which larger lipide aliquots have to be used. Lower recoveries have been observed with kidney lipide hydrolyses with use of only 20 ml. of $Ba(OH)_2$. We have analyzed all extracted aqueous phases for choline by the method previously reported (2) as a hydrolysis control, since these extracts were the same as those used in the study of lipide hydrolysis for the determination of choline. In several instances not reported in Table I, hydrolysis was incomplete with 20 ml. of saturated $Ba(OH)_2$.

TABLE II

Total Sphingosine Content of Dog Tissues

Tissue	Total sphingosine per gm. dry lipide-free residue			Sphingosine N, per cent of total lipide N			Total sphingosine from cerebrosides and sphingomyelins of beef tissue*
	Dog 4	Dog 5	Dog 6	Dog 4	Dog 5	Dog 6	
	μM	μM	μM				
Cerebrum		331	426		32.8	35.3	436
Skeletal muscle		8.13	6.3		12.1	8.7	17.0
Pancreas	22.5	24.6	19.6	12.7	11.6	10.7	
Kidney		39.4	30.2		19.1	12.6	36.5
Heart	17.5	19.7	16.7	14.8	13.1	11.7	37.5
Intestine	24.1	21.6	23.7	19.3	17.1	17.0	22.8
Liver			9.3			8.2	11.4
Spleen	29.3		25.5	19.5		17.6	
Lung	42.2		32.3	21.2		18.3	41.0

* Calculated from the data of Kaucher *et al.* (9); expressed as average micromoles per gm. of dry lipide-free residue.

The duration of acid hydrolysis may or may not be critical. The $1\frac{2}{3}$ hour hydrolysis recoveries have always been satisfactory; however, our data do not prove that a $\frac{2}{3}$ hour hydrolysis is unsatisfactory. Whether or not the acid hydrolysis is necessary at all would, of course, depend on the partition of psychosin between $CHCl_3$ and water. It would certainly be expected to be more soluble in the acid aqueous phase than sphingosine itself. For routine determinations we are arbitrarily using $1\frac{2}{3}$ hour hydrolyses.

A summary of analyses of dog tissue lipide extracts for total sphingosine is reported in Table II. These extracts were previously analyzed for total lipide nitrogen, phosphorus, and choline (2). Sphingosine is therefore expressed in terms of micromoles per gm. of dry lipide-free tissue.

DISCUSSION

Several factors must be considered in evaluating this method for the determination of sphingosine. The specificity of the method for the sphingosines alone depends on two assumptions: (1) that lipide extracts prepared and purified as described (1) are free of impurities which would contribute nitrogen to chloroform extracts of their hydrolysates, and (2) that tissue lipides contain no bases other than sphingosine which are extractable with chloroform from aqueous solutions at low pH. The first assumption is most probably valid because a minimum of nitrogenous impurity remains in these purified extracts. Also, after prolonged hydrolysis the breakdown products of most nitrogenous impurities would probably exist in ionic state at the extraction pH and not pass into the chloroform layer. The second assumption holds for all the nitrogenous substances in lipides which have been characterized. However, the "neuraminic acid" isolated by Klenk (6) as a component of gangliosides contains primary amino nitrogen. Whether this substance or its hydrolysis breakdown products, if there are any, would be chloroform-extractable cannot be stated. Only an amine of predominantly hydrocarbon character such as sphingosine would be expected to show preference for solvents over water with its amino group charged.

In this connection it may be noted that the spleen lipides of Dogs 4 and 6 contained only 19.5 and 17.6 per cent of sphingosine nitrogen, whereas the nitrogen to phosphorus ratio of these extracts was 1.31. If sphingosine were to account for all "extra" nitrogen over a N:P ratio of 1.00, then the percentage of sphingosine nitrogen should have been 23.6 per cent of lipide nitrogen. This discrepancy greatly exceeds the error of the methods. Hence, another nitrogenous substance is present which *is not included in the sphingosine determination.* Since beef spleen contains gangliosides (7), it is probable that they are also present in dog spleen and the neuraminic acid component may be the source of at least part of this extra nitrogen. In general, however, the lipide extracts of the other tissues with high N:P ratios have sphingosine contents approximating the "extra" nitrogen over the ratio of unity.

Dihydrosphingosine would be expected to accompany sphingosine in the chloroform extraction of the acid hydrolysate and both forms of sphingosine should, therefore, be determined by this method. However, the matter cannot be considered settled until data are obtained with pure dihydrosphingosine.

The actual determination of nitrogen in the chloroform extracts is somewhat inconvenienced by the fatty acids present and subject to the usual mechanical objections to all test-tube digestion analyses. However, we have found reliability in triplicate analyses on each extract and the sensitivity of the method described should make it useful. In our hands the average deviation from the mean nitrogen value is usually not over 2.5 per cent and is occasionally as low as 1.5 per cent. The analysis of the chloroform extracts for nitrogen by a Kjeldahl type of digestion and distillation should increase the precision of the method, but would greatly increase the amount of lipide extract needed for the determination.

Sphingosine might also be determined by the chromic acid oxidation described by Bloor (8) for the determination of fatty acids. We have found that this reagent oxidizes sphingosine and triacetylsphingosine completely to CO_2, H_2O, and NH_3. The oxidation is quantitative and reproducible when a 45 minute oxidation period is used. However, fatty acids would have to be removed before such a measurement of sphingosine could be applied to lipide hydrolysates. It may be noted that sphingosine would be a contaminant in the determination of fatty acids by this method, since the acids are usually extracted from acidified hydrolysates. The error thus introduced would be considerable in the determination of fatty acids in some tissues.

Expression of concentration of the various lipide bases in terms of micromoles per gm. of dry lipide-free residue has been taken as a course of accuracy, convenience, and for a more significant method of expressing results. It greatly expedites the quantitative manipulation of frozen fresh tissue powder, since only the tissue which is extracted contributes lipide material and is weighed as the residue. It is essentially an expression of moles of lipide base per gm. of cellular metabolic substance and automatically rules out artifacts resulting from changes in dry weight or neutral fat in a tissue.

The results obtained from the application of this method to the analysis of dog tissue lipides cannot be directly compared with results from other methods. However, one can roughly compare them with the sphingosine equivalent of the sum of the glycolipides and sphingomyelins found in beef tissues by Kaucher *et al.* (9). In their study glycolipide was determined by measurement of galactose by reduction, and sphingomyelins by precipitation as the reineckate and determination of phosphorus on the precipitate. Since total lipide as per cent of dry weight of tissue was also given in this report, one can calculate the micromoles of cerebrosides and sphingomyelines per gm. of dry lipide-free residue in the several tissues. Data from this study may thus be compared with our own (Table II). The magnitude of the sphingosine contents is generally comparable. Differences may be ascribed to species differences as well as to those arising from analytical methods.

The absolute amount of sphingosine per gm. of dry lipide-free tissue residue was found to vary considerably from dog to dog. This was also the case with nitrogen, phosphorus, and choline. However, the data show less variation in the per cent sphingosine nitrogen of total lipide nitrogen. As was suggested in the case of choline, nitrogen, and phosphorus, it would appear that the composition of the tissue lipides is fairly constant in normal animals, although the absolute amount per gm. of dry tissue residue may vary.

SUMMARY

A method is described for an approximate determination of sphingosine in whole lipide extracts which have been freed of non-lipide impurities. The method is based on a chloroform extraction of the aqueous lipide hydrolysate which is both specific and relatively quantitative for sphingosine. Sphingosine is then determined on the chloroform extract as nitrogen. The method has been used routinely in the range of 6 to 20 μM of sphingosine with triplicate nitrogen analyses. Sphingosine nitrogen comprises from 8 to 35 per cent of total lipide nitrogen in the tissues analyzed.

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THE SYNTHESIS OF 17-(METHYL-C^{14})-TESTOSTERONE AND 21-C^{14}-PROGESTERONE

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The use of steroid hormones containing tagged atoms promises to have an important part in the study of their metabolism. The relative availability of radioactive C^{14} manufactured in the uranium chain-reacting pile at Oak Ridge has facilitated the synthesis of such compounds containing labeled carbon atoms (1). Through the cooperation of Dr. Konrad Dobriner of the Sloan-Kettering Institute for Cancer Research we have been able to synthesize two important members of this group of substances, methyltestosterone and progesterone, having the radioactive atom in the side chain.

Since the radioactive carbon was available to us in the form of methyl iodide, both syntheses used Grignard reactions. The usual procedure for the preparation of methyltestosterone is to start with dehydroisoandrosterone acetate and to use a large excess of methylmagnesium iodide. Obviously this method was not suitable for our synthesis. We, therefore, used the method of Miescher (2) in which androstene-3,17-dione 3-enol ether was allowed to react with 1 mole of the Grignard reagent. This proved to be a satisfactory method, giving in one step a yield of 42 per cent of purified product based on the methyl iodide used.

Our synthesis of progesterone was based on the general method for the preparation of ketones developed by Gilman and Nelson (3, 4). In this method the desired organocadmium derivative is made from the corresponding Grignard reagent and then allowed to react with an acid chloride. This has been applied to steroid syntheses with good success (5, 6), and seemed to us to afford the most suitable method for the synthesis of progesterone. In spite of the fact that a large excess of Grignard reagent is usually used, we were able to obtain a yield of 50 per cent of pregnenolone acetate with only equimolecular quantities of methyl iodide and 3-acetoxy-Δ^5-etiocholenic acid chloride. Subsequent hydrolysis to pregnenolone followed by an Oppenauer oxidation gave an over-all yield of 26 per cent of purified product based on the methyl iodide used.

EXPERIMENTAL

17-(Methyl-C^{14})-testosterone—The methylmagnesium iodide was prepared from 1.34 gm. of C^{14}-methyl iodide containing 1.14 millicuries essentially as described by Tolbert (7). Our vacuum system was somewhat simpler than his and we used dry ice baths instead of liquid nitrogen. An equivalent of magnesium was placed in the reaction flask and the tube containing the frozen methyl iodide was sealed to the vacuum system. The apparatus was then evacuated with an oil pump, the system closed off, and the methyl iodide distilled into the reaction flask. This was then removed from the vacuum system while cold, and an addition funnel was attached. About 15 ml. of dry ether were admitted to the reaction flask and on warming to room temperature the Grignard reaction took place. After refluxing for a half-hour to complete the reaction, a solution of 2.96 gm. of Δ^4-androstene-3,17-dione enol ethyl ether (8) in 15 ml. of dry benzene was added. The mixture was then refluxed for 3 hours. The Grignard complex and the enol ether were hydrolyzed by adding 25 ml. of 10 per cent sulfuric acid and stirring at room temperature for 2 hours. The benzene-ether layer was separated and the aqueous phase extracted twice with ether. The combined extracts were washed with dilute sodium bicarbonate solution and water, dried, and the solvents removed. The crystalline residue was recrystallized from a small amount of ethyl acetate. The mother liquor fraction was chromatographed on alumina in benzene solution and the fraction eluted with 50 per cent benzene-ether yielded additional crystalline material. This was combined with the first fraction and recrystallized again from ethyl acetate. In this way we obtained 1.24 gm. (42 per cent) of material melting at 163–164°, $[\alpha]_D^{25} = +87°$ in ethanol. This material showed no melting point depression with a known sample of methyltestosterone and gave the same infra-red absorption spectra.

21-C^{14}-Δ^5-Pregnene-3-ol-20-one Acetate—The Grignard reagent in this synthesis was prepared as described above by using 726 mg. of C^{14}-methyl iodide containing 2 millicuries. After the reaction was complete 5 ml. of dry ether and 1.20 gm. of dry cadmium bromide were added with stirring. This mixture was refluxed for 2 hours, during which time the stirring was continued. The heat was then removed and the acid chloride from 1.82 gm. of 3-acetoxy-Δ^5-etiocholenic acid dissolved in 5 ml. of dry benzene was added in small portions. After the addition was complete the mixture was refluxed for 2 hours. It was then cooled and 4 ml. of water and 5.5 ml. of 10 per cent hydrochloric acid were added. The mixture was stirred for a half-hour and allowed to stand overnight.

The acid solution was extracted with ether and the extract washed with water, sodium bisulfite solution, 5 per cent potassium hydroxide solution, and finally with water. The potassium salt of the unchanged acid was relatively insoluble, so that its filtration from the extraction mixture was necessary. After drying the ether solution, the solvent was removed, leaving 930 mg. (50.6 per cent based on methyl iodide) of crude 21-C^{14}-pregnenolone acetate, melting point 138–144°.

21-C^{14}-Pregnene-3-ol-20-one—The above crude acetate was saponified by refluxing it for 2 hours in a solution of 25 ml. of methanol containing 1 gm. of potassium carbonate and 2 ml. of water. The mixture was concentrated *in vacuo*, diluted with water, and the steroid extracted with ether. The ether solution was washed well with water, dried, and the solvent removed, yielding 750 mg. (91.5 per cent) of crude pregnenolone, m.p. 165–175°.

21-C^{14}-Progesterone—The above material was oxidized directly by the Oppenauer method. It was dissolved in 50 ml. of dry toluene and 7.5 ml. of cyclohexanone were added. About 10 ml. of liquid were distilled to dry the system and then about 300 mg. of aluminum isopropylate were added. After refluxing for an hour, the solution was cooled, water was added, and the mixture steam-distilled to remove the toluene and cyclohexanone. It was finally concentrated to dryness, dilute hydrochloric acid added, and the progesterone extracted with ether. The ether solution was washed, dried, and the solvent removed, leaving 530 mg. of crude crystalline material, m.p. 110–118°. This was further purified by chromatography and yielded 250 mg. of pure radioactive progesterone, m.p. 126–128°, 175 mg. of cruder material, and a non-crystalline oil. The over-all yield of usable material was 26.2 per cent, based on the methyl iodide used.

Radioactivity Determinations—The methyltestosterone had an activity of 2.7×10^2 millicuries per mole or 0.9 microcurie per mg. The progesterone had an activity of 4.3×10^2 millicuries per mole or 1.4 microcuries per mg. The determination was made in the following way. The steroid after appropriate dilution was burned and the carbon dioxide collected in barium hydroxide solution. After washing and drying, the precipitated barium carbonate was converted to carbon dioxide and the absolute activity of the gas measured with the carbon dioxide-carbon disulfide mixture reported by Miller (9) and the apparatus described by Eidinoff (10). The relative activity by this procedure is reliable within 2 per cent and the absolute activity is accurate within 10 per cent.

In a recent paper Riegel and Prout described a similar synthesis of 21-C^{14}-progesterone (11).

We wish to express our thanks to Professor Melvin Calvin and Dr. B. M. Tolbert of the University of California for the radioactive methyl iodide. We are also greatly indebted to Dr. M. L. Eidinoff of the Sloan-Kettering Institute for Cancer Research for making the radioactive determinations through the generous cooperation of Dr. T. F. Gallagher.

SUMMARY

We have synthesized two steroid hormones, methyltestosterone and progesterone, with radioactive C^{14} in the side chain.

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THE SYNTHESIS OF PROLYLCYSTEINYLTYROSINE

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Although cysteine-containing peptides are of considerable interest to the biochemist, relatively few such compounds are known. The biologic significance of these substances is not limited to the oxidation-reduction system of the sulfhydryl-disulfide linkage, but cysteine- or cystine-containing peptides and proteins are of considerable pharmacologic interest. Among such compounds may be mentioned insulin and at least two of the hormones of the posterior pituitary, the oxytocic and the pressor principles.

During a systematic investigation of the oxytocic principle, it became necessary to prepare model compounds for chemical degradation experiments, as well as for studies involving the action of enzymes and reaction kinetics. A model compound of this type must meet certain requirements; *e.g.*, it must be relatively easy to prepare in adequate amounts and must show some similarity to the compound that it is supposed to represent. This implies that it contains, most, if not all, of the amino acids of the oxytocic principle. Hence, known peptides containing cysteine or cystine would not be suitable for this purpose.

The best available evidence regarding the amino acid composition of the oxytocic principle points to the presence of arginine, cysteine, proline, leucine, and tyrosine. Using purified preparations, Stehle and Trister (1) isolated tyrosine as the picronolate, arginine as the flavianate, proline as the reineckate, and leucine as its hydantoin from hydrolysates of the oxytocic principle. The presence of tyrosine and cysteine has been demonstrated by du Vigneaud *et al.* (2) and confirmed by others (3–7). Since synthetic methods whereby arginine could be introduced into an endo position are not very satisfactory, and since the introduction of leucine was expected to contribute little to the anticipated degradation studies, our attention was directed toward the synthesis of a tripeptide containing cysteine, proline, and tyrosine.

Of the six possible combinations in which these three amino acids may exist in a peptide chain, only those containing cysteine in the *endo* position were thought to be of particular interest. These are prolylcysteinyltyrosine and tyrosylcysteinylproline, either of which would serve the purpose equally well. The choice between them would depend upon the ease of preparation.

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Tyrosylcysteinylproline would have to be prepared through the intermedium of the unstable proline ester, whereas no difficulty was anticipated in the synthesis of prolylcysteinyltyrosine. The present communication, therefore, deals with the preparation of this compound by well established, synthetic methods.

Carbobenzoxyprolyl chloride was prepared by the method of Abderhalden and Nienburg (8) and condensed with the ethyl ester of S-benzylcysteine. In the original attempt, 2 moles of free ester were used per mole of acid chloride in order to neutralize the free hydrochloric acid liberated as the reaction proceeded. Under these conditions, solubility relations were such that the product was heavily contaminated with ester hydrochloride, a complication which could be avoided by the use of slightly more than 1 equivalent of pyridine for each mole of acid chloride. Pyridine hydrochloride precipitated from the solution as the reaction proceeded, and thus only 1 equivalent of free ester was required. Satisfactory yields were obtained and the quality of the product was greatly improved.

Carbobenzoxyprolyl-S(benzyl)-cysteine ethyl ester could be saponified to the free acid, but the corresponding acid chloride proved unsatisfactory for further synthetic operations. By the alternate route, the ester was transformed into the hydrazide, which was readily soluble in dilute acetic acid, the solvent of choice for the formation of the azide. This azide was allowed to react with free tyrosine ethyl ester in ethyl acetate solution to give carbobenzoxyprolyl-S(benzyl)-cysteinyltyrosine ethyl ester which, after saponification in aqueous solution, was treated with sodium in liquid ammonia. This treatment resulted in the simultaneous removal of the carbobenzoxy and benzyl groups leading to the free, reduced tripeptide. Final purification was accomplished by means of the copper salt in a manner similar to that employed for glutathione (9). The tripeptide crystallized readily from dilute alcoholic solution (Fig. 1).

EXPERIMENTAL

*Carbobenzoxy-*L*-prolyl Chloride*—This compound was prepared according to the procedure of Abderhalden and Nienburg (8).

*Carbobenzoxy-*L*-proplyl-S(benzyl)-*L*-cysteine Ethyl Ester*—40 gm. of S-benzylcysteine ethyl ester hydrochloride were dissolved in 200 cc. of water and the solution was cooled in ice. Sufficient 20 per cent sodium carbonate was then added to liberate the free base quantitatively. The product separated as a heavy oil that was extracted with ethyl acetate; the extracts were dried and concentrated to 100 cc. 20 cc. of anhydrous pyridine were then added to this solution with exclusion of moisture and the mixture was cooled in ice. With rapid stirring and continued cooling, a solution of 18.1 gm. of carbobenzoxy-L-prolyl chloride in 200 cc. of absolute ether was added, drop by drop, over a period not exceeding 15 minutes. Stirring was continued for another hour at 0° and the mixture allowed to stand at room temperature for an additional hour.

The reaction mixture was then poured into 500 cc. of 5 per cent hydrochloric acid. The ethyl acetate layer was separated and washed with water and sodium bicarbonate, dried over magnesium sulfate, filtered, and evaporated to dryness under reduced pressure. The residue crystallized instantly. After recrystallization from dilute alcohol, ether, and petroleum ether-ether mixture, the compound melted at 75–77°. 12.3 gm. of the pure product were obtained.

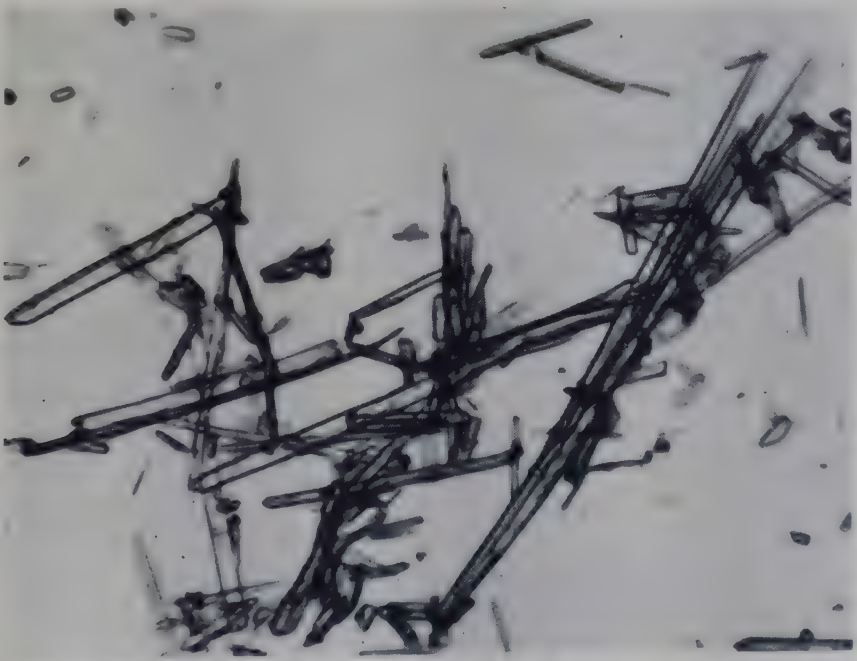

FIG. 1. Photomicrograph of prolylcysteinyltyrosine × 360

$C_{25}H_{30}O_5N_2S$.	Calculated.	C 63.83, H 6.38, N 5.96, S 6.81
470	Found.	" 63.74, " 6.34, " 6.15, " 6.59
		" 63.76, " 6.28, " 6.05, " 6.51

Carbobenzoxy-L-prolyl-S(benzyl)-L-cysteine—500 mg. of the ester described above were ground in a mortar and suspended in an excess of 2 N alkali. The mixture was then warmed to 80° with agitation. The resulting clear solution was acidified and the precipitated product worked up in the usual manner. It was recrystallized from dilute alcohol, m.p. 128°; 250 mg. of pure product were obtained.

$C_{23}H_{25}O_5N_2S$.	Calculated.	C 62.55, H 5.67, N 6.35, S 7.25
441	Found.	" 62.40, " 5.80, " 6.38, " 7.58
		" 62.49, " 5.87, " 6.56, " 7.60

Carbobenzoxy-L-prolyl-S(benzyl)-L-cysteine Hydrazide—12.0 gm. of carbobenzoxy-L-prolyl-S(benzyl)-L-cysteine ethyl ester were dissolved in 200 cc.

of absolute ethyl alcohol. To this solution were added 25 cc. of hydrazine hydrate and the mixture was allowed to stand in the ice box for 72 hours. The crystals were collected and recrystallized from dilute ethyl alcohol, m.p. 139–140°. A total of 8.2 gm. of the pure product was obtained.

$C_{23}H_{38}O_4N_4S$ (456). Calculated, N 12.28; found, N 12.51, N 12.25

Carbobenzoxy-L-*prolyl*-*S*(*benzyl*)-L-*cysteinyl*-L-*tyrosine Ethyl Ester*—8.0 gm. of the above hydrazide were dissolved in 50 cc. of 40 per cent acetic acid. This solution was cooled in ice and 1.20 gm. (1 equivalent) of sodium nitrite, dissolved in 3 cc. of water, added. The azide precipitated as an oil and was extracted with cold ether. The ether extracts were dried with magnesium sulfate and added to 3.63 gm. of tyrosine ethyl ester (m.p. 105°) dissolved in 50 cc. of anhydrous ethyl acetate. After the mixture had stood at room temperature for 24 hours, a crystalline material had precipitated. 4.40 gm. of the crude product were obtained. This compound could be recrystallized from ether-ethyl acetate or acetone water mixture, m.p. 155–156°.

$C_{34}H_{39}O_7N_3S$.	Calculated.	C 64.40,	H 6.15,	N 6.63,	S 5.06
	Found.	" 64.20,	" 6.57,	" 6.93,	" 4.89
		" 63.98,	" 6.41,	" 7.12,	" 4.79

Carbobenzoxy-L-*prolyl*-*S*(*benzyl*)-L-*cysteinyl*-L-*tyrosine*—2.0 gm. of the tripeptide ester were finely ground in a mortar and shaken with an excess of 2 N NaOH at room temperature until most of it had dissolved. The cloudy solution was extracted with ethyl acetate, and the aqueous layer acidified with dilute hydrochloric acid. The free acid precipitated as an oil that crystallized upon standing in the ice box overnight. After several recrystallizations from dilute alcohol, the pure substance melted at 204°; 1.70 gm. of pure product were obtained.

$C_{32}H_{35}O_7N_3S$.	Calculated.	C 63.50,	H 5.78,	N 6.94,	S 5.28
	Found.	" 63.90,	" 5.98,	" 7.10,	" 5.07
		" 63.80,	" 6.08,	" 7.02,	" 5.09

Because of difficulties involved in accurate molecular weight determinations of the free tripeptide, this essential analysis was made at this stage of the synthesis.

Mol. wt., calculated, 605; found, 620, 631; 595, 618

L-*Prolyl*-L-*cysteinyl*-L-*tyrosine*—500 cc. of anhydrous liquid ammonia were collected in a 200 cc. three necked, round bottom flask cooled in a mixture of dry ice and acetone. 200 mg. of metallic sodium were required for the reduction of 1.70 gm. of carbobenzoxyprolyl-S(benzyl)-cysteinyltyrosine; *i.e.*, until a permanent blue color was obtained. 500 mg. of ammonium chloride were then added, which decolorized the solution almost immediately. Nitrogen was then blown through the mixture in order to avoid oxidation by atmospheric oxygen and to help in the spontaneous evaporation of ammonia. After 24 hours of this treatment, a small amount of white crystalline material remained in the flask with almost no residual ammonia. The residue was dissolved in 30 cc. of water, kept under a vacuum at room temperature for 2 hours, and then adjusted to a pH between 3 and 5 by the addition of dilute sulfuric acid. A suspension of freshly prepared cuprous oxide was added in small portions, the total amount of which was so adjusted that a slight excess was present at the end of the reaction. This mixture was stirred for 10 hours and then allowed to stand at room temperature for 24 hours. The precipitate was centrifuged, washed with water, and hydrogen sulfide was passed through its aqueous suspension. Following the removal of copper sulfide, the clear filtrate was concentrated under reduced pressure. A fine crystalline precipitate appeared when the volume was less than 10 cc. It was collected and recrystallized from water and dilute ethyl alcohol.

$C_{17}H_{23}O_5N_3S \cdot 2H_2O$.	Calculated.	C 48.90,	H 5.50,	N 10.05,	S 7.72,	H_2O 8.62
	Found.	" 49.29,	" 6.09,	" 10.13,	" 7.81,	" 8.50
		" 49.06,	" 6.20,	" 10.34,	" 7.68	

A solution of the crystalline tripeptide in constant boiling HCl containing no amino nitrogen and 0.640 mg. of total (Kjeldahl) nitrogen per cc. was refluxed for 24 hours. The total nitrogen remained unchanged and the amino nitrogen rose from 0.0 to 0.428 mg. per cc. Hydrolysis had therefore resulted in the formation of two new amino groups. This observation furnished additional evidence to substantiate the peptide nature of the compound.

SUMMARY

The polypeptide, prolylcysteinyltyrosine, has been synthesized by standard methods. The synthesis was accomplished by condensing carbobenzoxyprolyl chloride with S-benzylcysteine ethyl ester and transforming the condensation product into its hydrazide; its reaction with nitrous acid produced a soluble azide which could easily be condensed with tyrosine ethyl ester to give carbobenzoxyprolyl-S(benzyl)-cysteinyltyrosine ethyl ester. The free reduced tripeptide was obtained in good yield by removing the protecting groups with sodium and liquid ammonia.

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THE UTILIZATION OF GLYCINE FOR THE BIOSYNTHESIS OF BOTH TYPES OF PYRROLES IN PROTOPORPHYRIN

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Although it has been shown that glycine is the nitrogenous precursor of the protoporphyrin moiety of hemoglobin (1, 2), it has not been previously established that glycine is utilized for the formation of all four pyrrole structures of the porphyrin. In protoporphyrin (Fig. 1) two of the pyrrole rings (I and II) contain methyl and vinyl side chains and two (III and IV) contain methyl and propionic acid side chains; it is conceivable that these two different pyrrole structures are synthesized in the animal organism from different precursors. Degradation experiments were therefore undertaken to study the distribution of isotopic nitrogen in N^{15}-labeled heme, produced *in vivo* after the administration of N^{15}-labeled glycine, in order to determine whether glycine is used in the synthesis of both pyrrole types.

The degradation studies were carried out in a manner to give unequivocal data with respect to separation of the two different pyrrole structures found in protoporphyrin. Labeled hemin, obtained from the blood of a human and from the blood of ducks, after the administration of N^{15}-labeled glycine, was converted into hematoporphyrin dimethyl ether which was subsequently oxidized to yield methylmethoxyethylmaleimide (from pyrrole rings I and II) and methyl propionic acid maleimide or hematinic acid (from pyrrole rings III and IV) (Fig. 1). It can be seen from Table I that in each experiment the N^{15} values of the methylmethoxyethylmaleimide from pyrrole rings I and II and of the hematinic acid from pyrrole rings III and IV were equal, and were identical with the N^{15} concentrations in the porphyrin. Therefore, these experiments demonstrate that glycine is utilized equally for the formation of the two types of pyrroles in the protoporphyrin. These findings are in harmony with the hypothesis that in the biosynthesis of protoporphyrin a pyrrole ring is formed which is a common precursor of both types of pyrrole structures found in the protoporphyrin.

These studies do not as yet reveal the mechanism by which glycine is utilized in the biosynthesis of the porphyrin. However, this report, together with our previous work, demonstrated directly that the nitrogen of

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* United States Public Health Service Fellow for 1948. This report is from a dissertation to be submitted by Jonathan Wittenberg in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

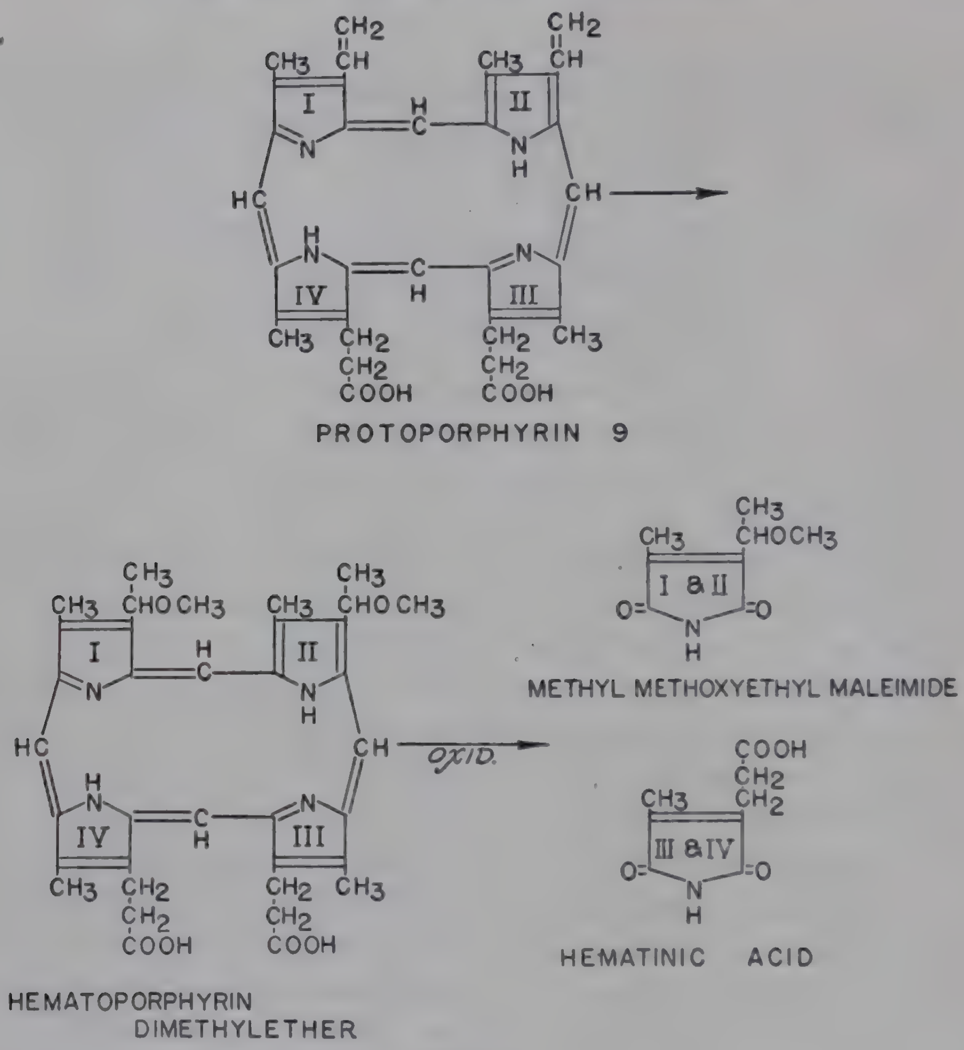
PROTOPORPHYRIN 9
OXID.
METHYL METHOXYETHYL MALEIMIDE
HEMATINIC ACID
HEMATOPORPHYRIN DIMETHYLETHER

Fig. 1

TABLE I

Distribution of N^{15} in Protoporphyrin

The results are expressed as atom per cent excess N^{15}.

Experiment	N^{15} concentrations in		
	Hematoporphyrin dimethyl ether	Methylmethoxyethyl-maleimide (pyrroles I and II)	Hematinic acid (pyrroles III and IV)
Duck	0.293	0.292	0.295
Human	0.113	0.112	0.113

glycine is utilized for the formation of heme. That the α-carbon atom of glycine is indeed utilized along with the nitrogen has been subsequently proved both by Altman *et al.* (3) and by Radin *et al.*[1] in the rat, and with the nucleated red cells of the duck (4).[1] On the other hand it has been demonstrated that the carboxyl carbon atom of glycine is not found in the tetrapyrrole, hemin, after the administration of carboxyl-labeled glycine to the dog (5) and to the rat[1] and on incubation with red blood cells of the duck.[1]

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[1] Radin, N., Rittenberg, D., and Shemin, D., unpublished results.

EXPERIMENTAL

N^{15} Hemin from Ducks—A solution of glycine containing 32 atom per cent excess N^{15} was injected intraperitoneally into two ducks. 1.5 gm. of glycine per kilo of body weight were administered in four injections spaced over a period of 2 days. 12 days later the birds were killed by exsanguination and from the pooled blood (175 cc.) 0.73 gm. of hemin was isolated by the usual method (6).

N^{15} Hemin from Human—A woman with polycythemia vera consumed at frequent intervals over a period of 2 days 48 gm. of glycine labeled with 32 atoms per cent N^{15} excess. For therapy blood was withdrawn 16 and 30 days after the start of the experiment. The hemin was isolated from the pooled samples of blood.

Preparation of Hematoporphyrin Dimethyl Ether—Hematoporphyrin dimethyl ether was prepared from the N^{15}-labeled hemin by a combination of the methods of Küster and Maurer (7) and of Fischer and Orth (8). 2.5 gm. of hemin were dissolved in 63 gm. of a solution of anhydrous hydrobromic acid in glacial acetic acid (density 1.41 at 0°) and stirred gently for 3 days at room temperature. The solution was taken to dryness *in vacuo* (bath temperature not over 50°) and the residue dried *in vacuo* over NaOH. The resulting crude dibromoporphyrin was dissolved in 23 cc. of absolute methanol, and 12.5 cc. of 20 per cent KOH in absolute methanol were added. After 12 hours at room temperature the solution was diluted with methanol, centrifuged, filtered, and taken to dryness *in vacuo* at 50°. The free porphyrin was isolated from the potassium salt by treatment with 25 per cent acetic acid and extraction into ether, evaporation, and storage *in vacuo* over NaOH. The yield of hematoporphyrin dimethyl ether, red-violet crystals, was 2.2 gm.; this was used for degradation experiments. A purer product can be obtained by dissolving in ether and concentrating to a small volume.

Oxidation of Hematoporphyrin Dimethyl Ether—The porphyrin was oxidized according to the method of Küster and Maurer (7). A solution of 4.62 gm. of chromic acid in a small amount of water was added with stirring over a period of 4 hours to a solution of 2.1 gm. of the porphyrin in 170 cc. of 20 per cent sulfuric acid. Stirring was continued for 18 hours, at which time the solution was green. The reaction mixture was diluted with water and extracted with ether for 4 to 5 hours in a continuous extractor. The ether solution containing both the methylmethoxyethylmaleimide and the hematinic acid was shaken once with 50 cc. of a 5 per cent sodium bicarbonate solution to remove the hematinic acid. The bicarbonate solution was then extracted six times with equal volumes of ether and the ether extracts combined with the original ether solution for the isolation of methylmethoxyethylmaleimide.

Preparation of Methylmethoxyethylmaleimide—The ether was removed by warming and the residue sublimed at 70–80° at 3 mm. of pressure. 0.52 gm. of crude crystals was obtained which were purified to constant melting point by recrystallizations from 15 to 20 cc. of petroleum ether (b.p. 70–90°). The yield of pure material was 0.36 gm.; m.p. 58.6–59.2° (Küster and Maurer (7) reported m.p. 58–59°).

$C_8H_{11}O_3N$.	Calculated.	C 56.8, H 6.6, N 8.3
Duck blood.	Found.	" 56.1, " 6.8, " 8.2
Human "	"	" 56.5, " 6.7, " 8.2

Preparation of Hematinic Acid—The bicarbonate solution from above was *immediately* made acid to Congo red paper with dilute sulfuric acid and after decolorization with a small amount of charcoal was extracted ten times with equal volumes of ether. The ether was removed *in vacuo* and the residue dried *in vacuo* over NaOH. The crude hematinic acid, 0.7 gm., was dissolved in ether and crystallized by the gradual addition of petroleum ether (b.p. 70–90°). Two recrystallizations usually sufficed to bring the material to a constant melting point. The yield was 0.46 gm.; m.p. 114–115°. (Küster (9) reported 113.5–114.5°.)

$C_8H_9O_4N$.	Calculated.	C 52.5, H 5.0, N 7.7
Duck blood.	Found.	" 52.7, " 4.9, " 7.7
Human "	"	" 52.3, " 5.1, " 7.6

SUMMARY

Glycine labeled with N^{15} was administered to ducks and to a human, and hemin was isolated from the red cells. The labeled hemin was degraded in order to study the isotope concentrations of the nitrogen atoms in the pyrrole structures of the porphyrin. The hemin was converted to hematoporphyrin dimethyl ether which was then oxidized to yield methylmethoxyethylmaleimide (derived from the methylvinylpyrroles of the porphyrin) and hematinic acid (derived from the methyl propionic acid pyrroles of the porphyrin). The N^{15} concentrations were found to be equal in the porphyrin, in the methylmethoxyethylmaleimide, and in the hematinic acid. These data demonstrate that glycine is the precursor for both types of pyrroles in the protoporphyrin molecule.

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CHROMATOGRAPHY OF AMINO ACIDS ON STARCH COLUMNS. SOLVENT MIXTURES FOR THE FRACTIONATION OF PROTEIN HYDROLYSATES

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In previous communications (1–3) procedures have been described for the quantitative separation of amino acids by chromatography on starch. The present paper is concerned with the extension of these techniques to include most of the amino acids commonly found in protein hydrolysates. In the earlier experiments *n*-butyl alcohol-benzyl alcohol solvents containing about 15 per cent water were employed to separate phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. In all alcohol-water solvents these are among the fastest moving amino acids on starch columns. Preliminary experiments had indicated (1) that the amino acids with slower rates of travel could be eluted successfully from the column by the appropriate choice of acidic solvents of higher water content. Many types of solvents have subsequently been investigated in order to arrive at a convenient system for the fractionation of protein hydrolysates.

The effluent concentration curves shown in Figs. 1 and 2 give the results obtained with two of the solvent mixtures which have proved most useful. The synthetic mixture of amino acids chromatographed corresponded in composition to a hydrolysate of bovine serum albumin. The effluent from the column was collected in a series of 0.5 cc. fractions on an automatic fraction-collecting machine (2). The amino acid concentration in each fraction was determined by the photometric ninhydrin method previously described (3). For the curve in Fig. 1, the column is started with a solvent composed of *n*-butyl alcohol, *n*-propyl alcohol, and 0.1 N HCl in the proportions of 1:2:1. After the emergence of aspartic acid, the rates of travel of the amino acids remaining on the column are increased by a shift of solvent to 2:1 *n*-propyl alcohol-0.5 N HCl. In this experiment, the first six amino acids are incompletely separated, and a chromatogram run with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water (2) is still required for resolution of these components.

The curve in Fig. 1 gives quantitative values for proline, threonine, aspartic acid, serine, glycine, ammonia, arginine, lysine, histidine, and cystine. Glutamic acid and alanine appear as a single peak. These two amino acids can be separated by the chromatogram illustrated in Fig. 2. The solvent in this case is composed of *tert*-butyl alcohol, *sec*-butyl alcohol, and 0.1 N HCl in the proportions of 2:1:1. Thus, by the use of three columns it is possible to separate from one another the eighteen constituents most commonly encountered in acid hydrolysates of proteins. The following experimental section describes the procedure employed to obtain results of the type shown in Figs. 1 and 2. The discussion deals with some of the considerations introduced by the presence of additional components

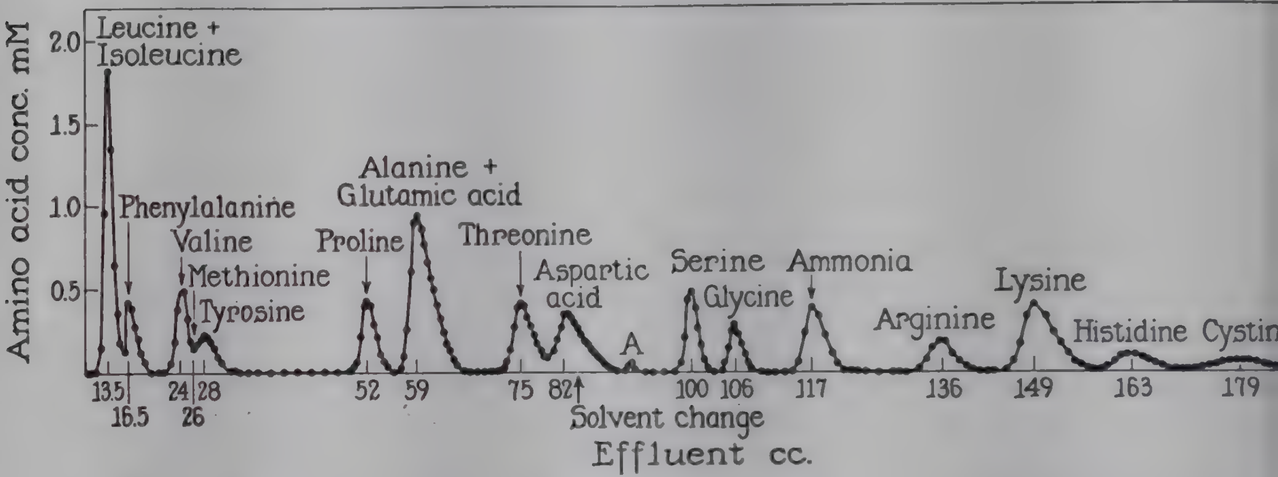

FIG. 1. Separation of amino acids from a synthetic mixture containing seventeen amino acids and ammonium chloride. Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl, followed by 2:1 *n*-propyl alcohol-0.5 N HCl. Column, 13.4 gm. of starch (anhydrous); diameter, about 0.9 cm.; height, about 30 cm. Sample, about 3 mg. of amino acids. *A* is a small artifact peak (see the text).

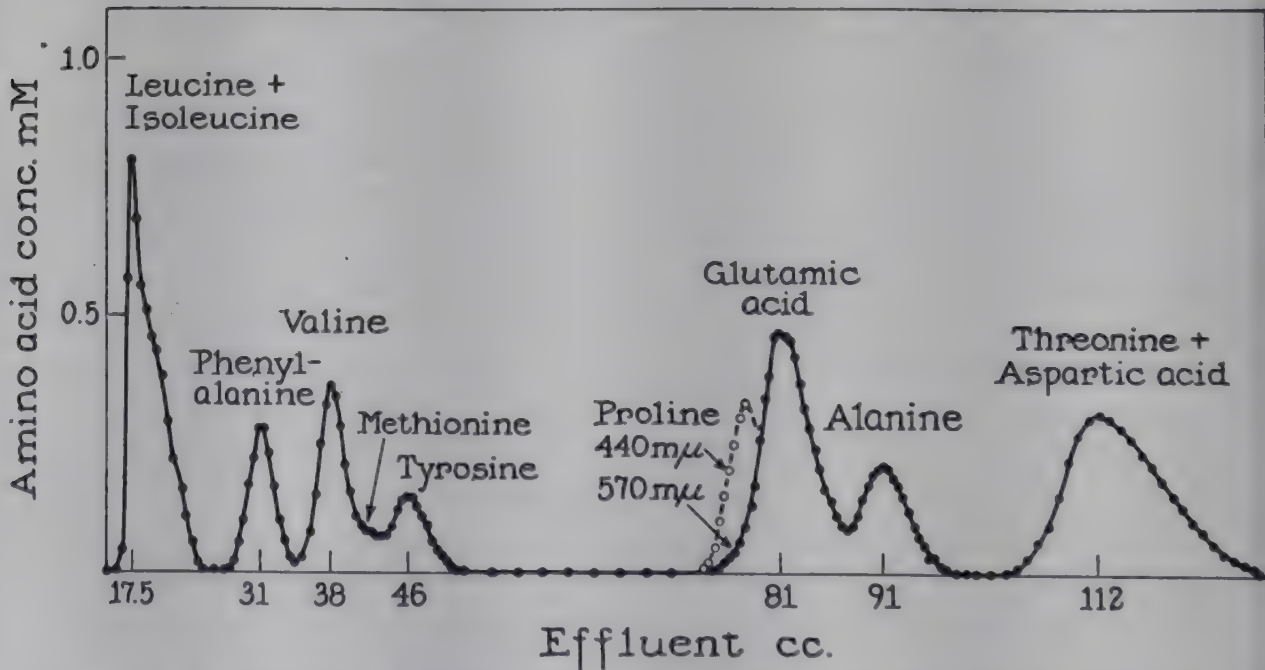

FIG. 2. Separation of glutamic acid, alanine, and other amino acids from a synthetic mixture containing eighteen components. Solvent, 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl.

in the mixture being fractionated and outlines the results obtained by the use of other solvent combinations. The results of analyses of hydrolysates of β-lactoglobulin and bovine serum albumin are given in the following paper (4).

EXPERIMENTAL

Preparation of Column—The potato starch column is poured as previously described (2).[1] Unless otherwise specified, starch columns 0.9 cm. in diameter and about 30 cm. in height have been used. The procedures can be scaled up proportionately for columns up to 8 cm. in diameter. After the starch has settled to constant height, the excess butyl alcohol is removed and a 1:1 mixture by volume of *n*-propyl alcohol-0.5 N HCl is placed on the column. The solvent is run through the column under a pressure of 8 cm. of mercury overnight, and the pressure is then raised to 15 cm. The solvent flow is continued until 0.5 cc. samples (neutralized) of the effluent and the influent solvent both yield the same color value when analyzed by the ninhydrin method. Starch contains small quantities of ninhydrin-positive material which are extracted by acidic alcohol solvents. The use of a propyl alcohol-HCl mixture with a 50 per cent water content serves to clean out the column fairly rapidly. When the number of cc. of the effluent required to yield a ninhydrin-negative column have been determined for a given batch of starch, the prescribed number of cc. can be used in the preparation of subsequent columns. For the samples of starch tested thus far, 55 cc. of the 1:1 solvent have proved adequate for columns 0.9 × 30 cm.[2] When the column is ninhydrin-negative, the solvent mixture is changed to that to be used in the chromatographic analysis.[3] After the new solvent has been run through overnight at 15 cm. pressure (20 to 25 cc. of effluent), the column is equilibrated and ready for use. Columns may be left in contact with solvents of low acidity, such as those prepared from 0.1 N HCl referred to in Figs. 1 and 2, for about 2 weeks before use without deterioration. Prior to the addition of the sample, the surface of the column is packed as previously described (2). When acidic solvents are used, there is no need for the 8-hydroxyquinoline treatment, which has been shown to be essential when neutral solvents are employed (2). It is desirable to run about 0.5 cc. of solvent into the freshly packed surface before the addition of the sample.

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[1] For work with acidic solvents, the delivery tip of the chromatograph tube can be pulled down so that a drop of effluent collects therein. In this manner ammonia from the air is prevented from reaching the inner walls of the tip. Beveled tips are still required on tubes which will be used with water as the solvent. If a beveled tip is used with acidic solvents, the inside section, up to the sintered plate, must be rinsed with a stream of the solvent before the column is placed on the fraction collector. A pipette, the end of which has been bent to form a U, is used for the rinsing.

[2] It is possible to wash large amounts of starch at one time with the propyl alcohol-HCl solvent, thus avoiding the preliminary washing each time a column is poured. This procedure is not recommended, however, since samples of starch washed and dried in the laboratory have been found not to give as uniform columns as the untreated commercial material (2).

[3] The solvents employed in these investigations have been prepared from *n*-butyl alcohol (reagent grade, Merck) and *n*-propyl alcohol, *sec*-butyl alcohol, and *tert*-butyl alcohol (C.P. grade, Columbia Organic Chemicals Company, Inc., Columbia, South Carolina). Redistillation prior to use has not been found necessary.

Addition of Sample to Column—The synthetic mixture of amino acids employed in the experiments shown in Figs. 1 and 2 was made up to simulate an acid hydrolysate of bovine serum albumin. To a total of about 1 gm. of amino acids in a 10 cc. volumetric flask, 1.5 cc. of 6 N HCl were added and the solution diluted to volume with water. A 0.5 cc. aliquot of this solution was diluted to 10 cc. with the solvent to be used in the chromatogram. A 0.5 cc. aliquot of the final solution, corresponding to 2 to 3 mg. of the amino acid mixture, was placed on the column and washed in as described earlier (2). In the developmental work on the placement of the peaks, simpler mixtures containing only a few components were similarly prepared. The pipettes should be calibrated for delivery both with water and with the organic solvent mixture.

Collection of Effluent Fractions—The delivery tip of the chromatograph tube is cleaned with a moist cloth and the column is placed on the automatic fraction collector (2). The pressure is maintained at 15 cm. and 0.5 cc. fractions are collected. The flow rate on a properly packed column should be 1.25 to 1.50 cc. per hour.

The use of propyl and *tert*-butyl alcohols on the fraction collector introduces problems which were not encountered with the butyl or benzyl alcohol solvent mixtures investigated earlier. When 0.5 cc. samples of the more volatile alcohol mixtures are allowed to stand on the machine overnight, there is considerable evaporation from the tubes. The loss in volume is not important, since the entire fractions are used in the ninhydrin analysis. But it has been noted that propyl alcohol-water mixtures, for example, have a marked tendency to creep up the glass walls of the photometer tube during the process of evaporation. Within 18 hours the solvent may creep almost to the top of the tube. The process can be observed by dissolving a few crystals of methyl red in 0.5 cc. of 2:1 *n*-propyl alcohol-0.5 N HCl. The quantity of amino acid which is carried to the upper portions of the tube as the solvent evaporates may comprise 4 to 8 per cent of the total amount present. This material is not in contact with the ninhydrin reaction mixture during the analytical determination. Hence, the recoveries of amino acid, under these conditions, run low.

It has been found that the creeping of volatile alcohols can be completely eliminated by rendering the glass surface hydrophobic by means of a silicone film. Glassware coated with a silicone film is repellent to water and to the water-miscible alcohols such as propyl alcohol and *tert*-butyl alcohol. For the present experiments all the sets of photometer tubes (3) for use with the fraction collector have been coated with Dri-film No. 9987 (General Electric Company, Schenectady, New York), which is a mixture of organochlorosilanes. For polymerization on glass, the Dri-film is applied as a 5 per cent (by volume) solution in chloroform (reagent grade). The coating of the tubes should be carried out in a hood and gloves should be worn. The glassware is first cleaned in chromic-sulfuric acid cleaning solution, thoroughly rinsed, dried at 110°, and allowed to stand at room temperature for 1 hour. When sets of 200 tubes are being coated, 200 cc. of the Dri-film solution are prepared. A sheet of filter paper or a towel is placed on the bottom of each test-tube rack (3). The first ten tubes are filled about half full with the filming solution. Each tube is emptied rapidly over a flask or beaker, causing the solution to flow over the upper walls of the tube, and set to drain inverted in the rack. The filming solution is used over again for the treatment of 200 tubes. The racks are left at room temperature overnight. The tubes are then returned to the upright position and each rack is baked for 2 to 3 hours in an air oven at 150–180°. This procedure has given more durable silicone films than those obtained by applying the Dri-film in vapor form or by the use of less concentrated solutions of the coating agent. Control of the relative humidity at which the filming is conducted has not proved necessary.

The film has no effect on the optical properties of the tubes as measured in the Coleman junior spectrophotometer. The silicone-coated tubes have maintained their water repellency during constant use for periods of about 6 months, at the end of which time recleaning and refilming have been necessary. The film is remarkably resistant to boiling water, alcohols, or acids, but is readily destroyed by alkali or cleaning solution. The coating is also rendered ineffective by ordinary soap, but Duponol C has been found to have no injurious effect. The washing procedure for the coated photometer tubes, therefore, is different from that previously described (3). After each set of ninhydrin analyses, the tubes are rinsed with water in racks of 50 and scrubbed with a brush (*e.g.*, E. Machlett and Son, New York, catalogue No. A-7-870) which has been dipped in a 0.2 per cent solution of Duponol C. The brushing is necessary to remove the ring of material that is sometimes deposited on the walls of the tubes. If this deposit resists removal by brushing, it is an indication that the tubes need refilming. An aluminum rod notched to fit the rim of the tube is useful for holding the individual tubes in position in the rack while they are being brushed. The brush employed should be reserved for this purpose and kept out of contact with ordinary soap. No evidence of any scratching of the photometer tubes by this cleaning procedure has been observed, but care should be taken to insure that no metal parts of the brush make contact with the walls of the tubes. The tubes are rinsed several times with distilled water and dried in an oven at 110°.

To prevent creeping of the solvent on the tip of the chromatograph tube and the glass funnel of the fraction collector, these items are also given a silicone coating. The tip of the chromatograph tube is cleaned with a hot mixture of HNO_3 and H_2SO_4 and coated by dipping the lower portion of the tube in the Dri-film solution, contamination of the sintered glass plate being avoided. The funnel of the fraction collector is coated both inside and outside.

In order to be certain of the proper setting for the impulse counter when a water-repellent tip is used, it is necessary to redetermine the drop size (2) more frequently than is required with an untreated funnel. For the solvents referred to in Fig. 1, the drop sizes have been so nearly the same that a single impulse counter setting has been used throughout the experiment.

The use of acidic solvents requires precautions against the uptake of ammonia from the air by the effluent fractions during the period they are standing on the automatic fraction collector. The ninhydrin method employed to analyze the effluent can readily detect 0.1 γ of ammonia per cc. If no preventive steps are taken, tubes containing 0.5 cc. of 2:1 propanol-0.5 N HCl, left overnight open to the laboratory air or on the fraction collector, may pick up enough ammonia to give a positive reading of 0.10 optical density unit in the ninhydrin analysis. This uptake may be virtually eliminated by lining the inside surface of the cover of the fraction collector with filter paper impregnated with citric acid. Large sheets of filter paper are cut to fit the cover and taped in position. A 2 per cent solution of citric acid in ethanol is brushed onto the surface. With the fraction collectors in use in this laboratory, the ammonia problem has been increased by the liberation of ammonia from the bakelite parts of the machines. It was not appreciated for some time that hexamethylenetetramine is used in the manufacture of many samples of bakelite and that the material, as a result, may contain appreciable quantities of ammonia. Samples of bakelite can readily be tested for ammonia liberation as described earlier (2). If the test is positive, the citric acid solution must be applied to all the bakelite parts of the fraction collector, including the phototube housing. Commercial models of the fraction collector are currently being built with special ammonia-free bakelite,[4] which eliminates this source of contamination.

In work with acidic solvents, the cotton packing around the stem of the chromatograph tube is also treated with citric acid. When the tubes are removed from the machine, they are stoppered with corks which have

[4] The Technicon Company, 215 East 149th Street, New York 51.

previously been shaken with the alcoholic citric acid solution and air-dried. Corks thus treated have been satisfactory for a year or more. Rubber stoppers have proved unsuitable.

Contamination with ammonia can also occur during the handling of the solvents. The lips of all storage vessels should be wiped before use. Care must be taken to avoid any liquid contact between the solvent and the rubber stoppers on the top of the column and the top of the separatory funnel. The glass should always be wiped dry before the insertion of the stoppers. It is important that the need for reimpregnation of the cover on the machine be checked periodically by placing test samples of the 2:1 *n*-propyl alcohol-0.5 N HCl solvent on the machine overnight. The ninhydrin readings should be no higher than those of control tubes which have remained stoppered prior to analysis.

In performing a chromatogram of the type referred to in Fig. 1, a solvent change is made about half-way through the experiment. The effluent fractions should, if possible, be analyzed each day to provide a check on the progress of the experiment and to furnish a basis for estimating the exact point at which the solvent change should be made. In an experiment such as that shown in Fig. 1, it is desirable to shift the column to the second solvent mixture during the emergence of aspartic acid. This point can be predicted fairly accurately by multiplying the position of the readily identified proline peak by 1.6. The change point is usually reached at about 83 cc. of the effluent and can be predicted from the position of one of the earlier peaks, if necessary. If the change of solvent is scheduled to occur at an inconvenient hour, the column can be slowed down by running it under lower pressure without affecting the results. At the time of the change of solvent, the separatory funnel is removed and the liquid above the starch in the chromatograph tube is withdrawn before the addition of the new solvent.

For the experiment illustrated in Fig. 1, the solvent shift occurs on about the 3rd day, and the completion of the experiment, through the emergence of cystine, requires about 7 days of continuous operation on the fraction collector.

When a column is shifted from one solvent to another, a specific series of changes occurs in the composition of the effluent. In the example shown in Fig. 1, the initial solvent contains 25 per cent water and is 0.025 N with respect to HCl. The second solvent contains 33 per cent water and is about 0.17 N with respect to HCl. The effluent attains the higher water content of the second solvent when a volume of solvent equivalent to that retained by the column has passed through the starch. The increased water content, which appears at about 6 cc. after the solvent change, serves to increase the rates of travel of the amino acids. If the solvent shift has been made too early, the latter part of the aspartic acid curve will be distorted. Since asymmetrical peaks frequently indicate the presence of more than one component, it is preferable, in order to avoid ambiguity, to arrange for the emergence of the higher water concentration after the aspartic acid curve is down to the base-line.

The increase in HCl concentration, however, to 0.17 N, occurs sharply at about 14 cc. after the solvent has been changed. The HCl thus has a "retention volume," in the terminology of Tiselius (5), of about 6 to 8 cc. The rise in the HCl content of the effluent in Fig. 1 occurs just at the beginning of the serine peak. Although a change in acid concentration is not capable of distorting the serine peak significantly, it is desirable from the analytical standpoint to have the change occur before the amino acid emerges.

The peak *A* in Fig. 1 is an artifact which occurs at the point of increase of the water content of the effluent. This small peak represents a transient rise of only 0.02 to 0.04 optical density unit in the blank and indicates that the starch column has been thoroughly freed of ninhydrin-positive material in the preliminary washing procedure. It is indicative also of the adequacy of the silicone film on the tip of the chromatograph tube and the funnel. In earlier experiments, before these parts of the glassware were coated, a relatively large artifact peak was usually obtained at position *A*. A control experiment with a strong solution of methyl red in the acidic solvent demonstrated that, during a 1 to 3 day run on unfilmed glassware, a small amount of solid material was deposited around the outside of the tip of the funnel as a result of creepnig and evaporation of the solvent. Similarly, a deposit of some of the solute could be seen around the periphery of the flowing stream of the effluent within the funnel. When the solvent was subsequently shifted to one of higher water content, and consequently different surface properties, some of this residue was redissolved and emerged as the artifact peak. A similar experiment with glassware rendered hydrophobic by a silicone film showed no residual deposit of methyl red on any part of the tip or funnel.

The shift from one solvent to another, after a sample has been added to a chromatogram, has proved practical only with solvents that are miscible with water in all proportions. When an attempt has been made to shift a butyl-benzyl alcohol column to a propyl alcohol-water solvent, droplets of water have formed at the interface, thus destroying the efficiency of fractionation (2).

Analysis of Effluent Fractions—The concentration of amino acid in the effluent fractions is determined by the photometric ninhydrin method (3). For the 0.5 cc. fractions, 2 cc. of the ninhydrin reagent are used. The solvents possessing a total acidity of 0.025 N or less do not require neutralization. Samples of the 2:1 *n*-propyl alcohol-0.5 N HCl mixture, however, must be neutralized just before the addition of the reagents. For routine work, a burette tip of appropriate size can be prepared to deliver 0.10 cc. of alkali per 2 drops. A rack of 50 tubes can conveniently be moved along underneath a burette dripping at a constant rate. The rack should be shaken by hand after the addition of the alkali. The concentration of NaOH (about 0.8 N) is adjusted so that, in the titration of test samples, 2 drops leave the fractions slightly acidic. The amount of alkali added should be such that an additional 0.1 cc. of 0.1 N NaOH is required to render the samples alkaline to phenolphthalein. One purpose of keeping the samples slightly acid is to avoid loss of ammonia from the NH_4Cl peak.

After a solvent shift, as in Fig. 1, it is necessary to locate the effluent fraction at which the increase in acid concentration occurs. A small drop of 0.02 per cent phenolphthalein in ethanol is added to the twenty-eighth fraction after the time of change of the solvent on the top of the column. Depending upon whether this tube is or is not rendered alkaline by 1 drop of the approximately 0.8 N NaOH, the tubes ahead or after it are treated similarly until the point is determined at which all subsequent fractions require 2 drops of the alkali. The subsequent additions are made without use of the indicator. By this procedure a few of the fractions around the change point may be overneutralized. No significant errors have been observed when the increase in acid concentration occurs during the first two or three fractions containing serine, but as already mentioned, it is preferable to have the change occur earlier.

For an experiment such as that illustrated in Fig. 1, every effluent fraction is analyzed until after the emergence of glycine. From that point on, analysis of every other fraction is sufficient. If the first chromatogram on an unknown sample shows areas in which there are no peaks, such as the long valley between tyrosine and proline in Fig. 1, the number of analyses required in a duplicate experiment can be reduced by omitting some of the fractions. If the chromatogram is being run for the determination of only one or two amino acids, the rest of the curve can be neglected. The solvent mixture referred to in Fig. 2 is usually employed only to separate glutamic acid and alanine, and generally the first 45 cc. of effluent are collected as a fore fraction before the column is placed on the fraction collector. If accuracy to the last few per cent is not important, the amount of ninhydrin required can be halved by the use of only 1 cc. of ninhydrin solution per 0.5 cc. sample.

The choice of blanks against which the amino acid peaks are read is crucial for maximum accuracy in the integration of the curves. In many instances the average blank tube for the base-line of the effluent curve can be readily determined in the manner previously outlined (2). In the first part of Fig. 1, there are blank tubes ahead of leucine and in the valleys before proline and threonine. The proline peak, reddish yellow in color, is read at 440 mμ. There is always the possibility, however, that a given group of tubes taken for ninhydrin analysis may not contain an adequate number of fractions from the blank sections of the curve. Therefore, three or more empty photometer tubes are added routinely to each rack of samples submitted to the ninhydrin analysis. The prescribed amount of ninhydrin solution is added to all the tubes. The reagent blank, obtained on the tubes which received only the ninhydrin solution, may vary slightly from day to day or with the batch of reagent solution. The reagent blank frequently amounts to 0.14 to 0.20 optical density unit when read against a reference tube of 1:1 *n*-propyl alcohol-water (3). The column blanks with the 1:2:1 solvent of Fig. 1 and the 2:1:1 solvent of Fig. 2 are usually not identical with the reagent blank, differing by perhaps 0.01 optical density unit. If there is no definite group of column blanks in the set being analyzed, the tubes can be read against the reagent blank. The readings can subsequently be corrected to a column blank by reference to the differential between the column blank and the reagent blank in the preceding or the following day's analyses.

The change of solvent to 2:1 *n*-propyl alcohol-0.5 N HCl introduces changes in the column blank. Following the emergence of the artifact peak (*A* in Fig. 1), the solvent of increased water content which is then emerging may give a reading that is 0.01 to 0.03 optical density unit higher than the reagent blank. An additional rise of 0.01 to 0.02 unit takes place at the point where the increased acid concentration appears in the effluent. The valley between serine and glycine does not always fall to the base-line, and the column blank for both of these peaks is, therefore, taken in the valley after glycine. The fractions before or after the ammonia peak provide the blank in this range. In order to obtain accurate values for ammonia, a standard solution of NH_4Cl and its appropriate blank should be run along with the column samples (3). The base-line for the arginine peak is taken from the valley between ammonia and arginine. The fractions between arginine and lysine usually return to this same value, but not invariably.

It has been found that quantitative recoveries of lysine, histidine, and cystine are obtained only when these amino acid peaks are read against the column blank taken after the emergence of cystine. The valleys in this range do not always fall to the base-line. Since cystine is the last amino acid to emerge, the proper blank is usually not available when the lysine and histidine peaks are analyzed. In this range, therefore, the tubes are read against the fraction giving the lowest reading or against the reagent blank as zero. If some of the tubes have been read against a fraction which gives a reading 0.05 optical density unit above the reagent blank, and the final column blank after cystine has dropped to 0.02 unit above the reference tubes, the correction is made by adding 0.03 unit to the appropriate fractions before integration of the peaks.

The variations in the blank and the need for the use of these corrections, however, mean that the accuracy of integration of the peaks after the solvent shift in Fig. 1 is, as a rule, not as satisfactory as that obtained in chromatograms developed with a single solvent mixture.

TABLE I

Ninhydrin Color Yields from Amino Acids in Organic Solvent Solutions

Determined on 0.5 cc. samples of 0.35 mM solutions of the amino acids. Heating time 20 minutes. The samples in 2:1 *n*-propyl alcohol-0.5 N HCl were neutralized with 0.1 cc. of about 0.8 N NaOH prior to analysis.

Compound	Color yield on molar basis relative to leucine in water, read at 570 mμ		
	1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl (d^{25} =0.862)	2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl (d^{25} = 0.858)	2:1 *n*-propyl alcohol-0.5 N HCl (d^{25} = 0.882)
Leucine	0.99	1.00	
Isoleucine	1.00	1.02	
Phenylalanine	0.85	0.85	
Valine	1.01	1.02	
Methionine	1.00		
Tyrosine	0.86	0.86	
Proline	0.05 (0.27 at 440 mμ)	0.05	
Glutamic acid	1.02	1.02	
Alanine	1.02	1.00	
Threonine	0.94		
Aspartic acid	0.89		
Serine			0.94
Glycine			0.98
Ammonia			0.98 *ca.**
Arginine			0.97
Lysine			1.14
Histidine			1.01
Half cystine			0.54

* For accurate ammonia determinations the factor should be checked with a known NH_4Cl solution run at the same time as the unknown (*cf.* (3)).

Calculations—The procedure for integration of the curves has been outlined earlier (3). When only every other effluent fraction is analyzed (*i.e.*, ammonia through cystine, Fig. 1), satisfactory recoveries are obtained by doubling the usual summation (*cf.* (3), Table V[5]). For the relatively volatile solvent mixtures referred to in Figs. 1 and 2, the entire 0.5 cc.

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[5] Table V (3) contains an error. In the third line of the integration below Table V, read "Sum of Fractions 37–40 and 45–47" for "Sum of Fractions 37–42 and 46–47."

sample evaporates during the 20 minute heating in the water bath. For unneutralized samples, the calculated correction factors for 5, 10, and 15 cc. of diluent (*cf.* (3), Table III) become 0.230, 0.216, and 0.212. For samples which have been neutralized with 0.10 cc. of NaOH, the factors are 0.232, 0.218, and 0.212. In the integration of the curves, the summations of the uncorrected amino acid concentrations are routinely multiplied by one-half the above factors (*cf.* (3), Table V[5]). The whole factors are used for the conversion of the analytical results to leucine equivalents in plotting the curves for publication and in the determination of ninhydrin color yields on standard solutions. If only 1 cc. of the ninhydrin solution is used per 0.5 cc. sample, the evaporation loss is about 0.62 cc. The factors are 0.193, 0.196, and 0.199 for unneutralized fractions and 0.196, 0.198, and 0.199 for samples neutralized with 0.1 cc. of NaOH.

The ninhydrin color yield for each of the amino acids has been determined in the solvent in which it emerges from the column. The yields given in Table I should be checked under the user's experimental conditions (3). It is convenient to prepare 2 mM standard solutions which are diluted to about 0.35 mM for analysis. The blanks consist of 0.5 cc. aliquots of the same sample of solvent.

Use of the color yield of 0.27 for proline at 440 mμ is the same as multiplying the leucine equivalents by 3.7, as previously described (3). For publication, the proline curve has been corrected, whereas the other peaks have been left in terms of leucine equivalents.

In most instances, the choice of fractions to be included in the integration of a given amino acid peak is evident from the graph of the results. In those cases in which the valley between two peaks does not fall to the baseline, one-half of the quantity of amino acid represented by the low point of the valley is assigned to each peak. This procedure has given satisfactory integrations when the valley is less than half the height of the smaller of the two peaks. In the present experiments, no pairs of peaks have been encountered which required an attempt to apply the method of calculation for overlapping components used in the case of tyrosine and valine in the butyl-benzyl alcohol solvent (2).

In the experiment shown in Fig. 2, proline overlaps glutamic acid. The entire glutamic acid curve is read at 570 mμ, and the integration subsequently corrected for the contribution of proline, which has a color yield of only 0.05 (relative to leucine as 1.00) at this wave-length (*cf.* (3)).

Quantitative Analysis of Synthetic Mixtures—The results obtained by the integration of the curves in chromatograms of the type shown in Fig. 1 are summarized in Table II. The synthetic mixture of amino acids corresponded in composition to an acid hydrolysate of bovine serum albumin. Cysteine was omitted since, as will be shown later (4), it was found not to be present in protein hydrolysates that had been repeatedly evaporated to dryness in order to remove excess HCl.

The separation of phenylalanine from leucine plus isoleucine is not sufficient to permit fully reliable division of the peaks. Since leucine and isoleucine are usually present in by far the larger quantity, the percentage recovery may be fairly accurate for these two amino acids. The phenylalanine values, although more variable, are frequently accurate to ±5 per cent. If the column is loaded more heavily, however, as is sometimes desirable in order to attain higher accuracy in the analysis for the basic amino acids, the resolution of leucine plus isoleucine and phenylalanine becomes less satisfactory than that indicated by Table II. Valine, methionine, and tyrosine are integrated as a group. On an unknown solution, the principal calculation of value for these combined peaks is the estimation of the total amino nitrogen in leucine equivalents.

TABLE II

Recovery of Amino Acids from Known Mixture Containing Eighteen Components

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl followed, after the emergence of aspartic acid, by 2:1 *n*-propyl alcohol-0.5 N HCl (*cf.* Fig. 1).

Constituent	Amount present	Per cent recovery			
		Chromatogram 456	Chromatogram 457	Chromatogram 520	Average
	mg.				
Leucine-isoleucine	0.364	99.4	99.5	101.5	100.3
Phenylalanine	0.165	94.8	96.1	94.8	95.2
Valine-methionine-tyrosine	0.354	99.6	101.0	100.1	100.2
Proline	0.136	99.7	97.8	100.0	99.2
Glutamic acid*-alanine	0.515	95.2	94.6	96.8	95.5
Threonine	0.201	97.5	101.0	102.0	100.2
Aspartic acid*	0.267	93.5	94.1	94.7	94.1
Serine	0.118	100.0	99.8	101.2	100.3
Glycine	0.051	99.1	100.5	101.0	100.2
Ammonia	0.024	102.0	99.5	104.5	102.0
Arginine	0.143	97.7	102.8	105.0	101.8
Lysine	0.302	96.3	103.0	99.5	99.6
Histidine	0.094	99.7	104.6	97.4	100.6
Cystine	0.133	89.5	102.7	101.5	97.9
All constituents	2.867	97.3	99.3	99.6	98.7

* When the value for glutamic acid is corrected for the 7 per cent low recovery due to esterification, the recoveries for glutamic acid plus alanine become 100.2, 99.7, and 101.7 per cent. The aspartic acid recoveries, which run 6 per cent low, may be similarly corrected to yield the figures 99.4, 99.9, and 100.8 per cent. The total recoveries, on this basis, become 98.6, 100.8, and 101.0 per cent.

Proline and threonine emerge as well defined peaks before the solvent shift and are recovered quantitatively. Adjacent to them, however, are the peaks for glutamic acid plus alanine and aspartic acid for which, it will be noted, the recoveries are low. It has been found that the yields of glutamic and aspartic acids are low in this solvent as a result of esterification. With unknown mixtures, the aspartic acid values obtained by integration are divided by 0.94 to give corrected figures.

The procedure which has been outlined for the establishment of the blank after the solvent shift permits quantitative recoveries to be obtained for the peaks emerging after the change to 2:1 *n*-propyl alcohol-0.5 N HCl.

The results obtained in the separation of glutamic acid and alanine with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl are summarized in Table III. Esterification of glutamic acid is negligible in this solvent mixture, as evidenced by the essentially quantitative recovery of the amino acid. The chromatogram also provides an alternative determination of phenylalanine, which is well separated in this case. If methionine is absent, the column can yield quantitative values for tyrosine and valine. In most instances, the column has been used only for the separation of glutamic acid and alanine. The valley does not fall to the base-line, and it sometimes is necessary to reduce the load on the column in order to obtain adequate resolution.

TABLE III

Recovery of Glutamic Acid, Alanine, and Other Amino Acids from Synthetic Mixture

Solvent, 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl (*cf*. Fig. 2). The mixture contained eighteen components (*cf*. Table II).

Constituent	Amount present	Per cent recovery			
		Chromatogram 474	Chromatogram 543	Chromatogram 481	Average
	mg.				
Leucine-isoleucine	0.373	99.0	100.8		99.9
Phenylalanine	0.169	101.6	103.6		102.6
Valine-methionine-tyrosine	0.363	100.6	104.4		102.5
Glutamic acid	0.426	96.3	97.8	100.2	98.1
Alanine	0.102	97.3	101.3	97.5	98.7

Accuracy of Chromatographic Analysis—In general, the chromatographic procedure on starch columns is capable of yielding recoveries of 100 ± 3 per cent. The average recoveries for the components of the synthetic mixture used in the chromatograms summarized in Tables II and III are well within this range. The deviations which do occur appear to be random and cancel out, in part, in the calculation of the total recovery for the sum of the amino acids, which is almost invariably accurate to ±1 per cent. In any given experiment, however, a number of factors operate to reduce the accuracy of the analysis for one or more of the constituents. The amount of a given amino acid present is the principal variable. When a mixture contains ten or twenty components, it is probable that a loading for the column which is optimum for some will not be the most favorable for all of the amino acids. When the optical density readings on the peak points of a curve are as low as 0.20, a variation of 0.01 unit in the blank can cause an error of 10 per cent in the integration. Some of the peaks integrated for Table II fall into this category. The accuracy of the recoveries indicates that, in practice, the averaging of a series of blanks usually establishes the base-line to considerably better than 0.01 optical density unit. But the determination is on a sounder basis if the load on the column can be increased to give a peak reading of 0.50 to 1.00 optical density unit. An increase in the total load, however, as has already been mentioned, can have an adverse effect upon the degree of resolution obtained in the case of components present in relatively large amounts. An attempt to obtain an adequate picture of the composition of a mixture in a single chromatogram will usually require a compromise on the question of the optimum load for the column. If the emphasis is on the determination of only a few specific constituents of the mixture, the load can be adjusted to give maximum accuracy for these amino acids. In the case of low peaks, it should also be possible to obtain increased accuracy by using 4 times the present sample size on a column 2 cm. in diameter, if 2 cc. effluent fractions are collected and concentrated to 0.5 cc. before analysis.

DISCUSSION

Identification of Amino Acid Peaks—A discussion has already been given (2) of the general measures which can be taken to assist in the identification of a peak on an effluent concentration curve. The problems associated with the interpretation of the results obtained with unknown mixtures were enumerated for the butyl-benzyl alcohol experiments (2) and apply with added emphasis to the present curves. The positions of the peaks shown in Fig. 1, together with the points of emergence of a number of additional amino acids[6] and related compounds, have been summarized in Table IV. The absolute value for the position of a peak is not as useful an aid in identification as it was in the case of simpler chromatograms. As in the earlier experiments, the relative positions of the peaks are highly reproducible. The same general pattern has been obtained routinely on both synthetic mixtures and protein hydrolysates. In a given experiment, however, all the peaks may emerge somewhat faster or slower than indicated by Table IV. Shifts of as much as 10 per cent have been obtained. These deviations can result from a number of causes, among which may be mentioned small variations in the amount of starch introduced during the pouring of the column, slight differences in the composition of the solvent mixtures, and errors in the adjustment of the size of the fractions collected.

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[6] We are indebted to Dr. A. Hiller and Dr. D. D. Van Slyke for a sample of hydroxylysine, to Dr. H. T. Clarke for a sample of cysteic acid, and to Dr. H. Borsook for a sample of α-aminoadipic acid.

TABLE IV

Order of Emergence of Amino Acids and Related Compounds

Determined on columns 0.9 × 30 cm. prepared from 13.4 gm. of starch (anhydrous), developed with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and shifted to 2:1 *n*-propyl alcohol-0.5 N HCl at 83 cc.

Compound (*cf.* Fig. 1)	Position of peak	Additional compounds	Position of peak
	cc.		*cc.*
Leucine-isoleucine	13.5	Diiodotyrosine	12.5
Phenylalanine	16.5	Tryptophan	18
Valine	24	α-Amino-*n*-butyric acid	38
Methionine	26	α-Aminoadipic acid	41
Tyrosine	28	Cysteic acid	64
Proline	52	Taurine	74
Glutamic acid-alanine	59	Hydroxyproline	80
Threonine	75	Sarcosine	84
Aspartic acid	82	Citrulline	98.5
Serine	100	Ethanolamine	102
Glycine	106	Asparagine	121
Ammonia	117	Glucosamine	126
Arginine	136	Histamine	160
Lysine	149	Ornithine	176
Histidine	163	Hydroxylysine	180
Cystine	179		

The exact point of the solvent shift, of course, affects the positions of the peaks after aspartic acid. These variations mean that, in a given chromatogram, a peak emerging at 163 cc., for example, cannot be stated to occur at the histidine position, unless it has been placed either by reference to the sequence of the other peaks from the sample or by observance of the rise of the peak at this position after the addition of known histidine.

Considerable variations have also been observed in the absolute positions of the peaks in Fig. 2. The deviations are believed to result from variations in the moisture content of the samples of *tert*-butyl alcohol from which the solvent has been prepared. If proline is present, its characteristic color in the ninhydrin reaction serves to identify the beginning of the glutamic acid peak.

The relative positions of the peaks are fully reproducible only if the column has not been overloaded. The amounts of each amino acid used for Fig. 1 are low enough so that the column is capable of yielding fairly symmetrical effluent curves. As the load of a given component is increased, a point is reached at which the peak in question begins to show a steep front, indicative of a non-linear isotherm. The tail portion of the peak is identical in position and slope with the right-hand half of the peak in Fig. 1, but the increased load will have advanced the point of maximum concentration 1 to 3 cc. ahead of its former position. If this trend is extended by increasing the load to 10 to 20 times the present level, the position of the advancing front is markedly moved ahead. In general, a 2-fold increase over the amounts given in Table II does not lead to significant distortion of the peaks, but, as already mentioned, the degree of increase in the load which is tolerable will depend upon the objectives of the given experiment.

The second column of Table IV gives the positions of some amino acids and related compounds not covered by Fig. 1. As the number of possible components in a mixture is increased, the problems of identification are multiplied, and no general solution can be offered. By the use of additional solvent mixtures, a number of the overlaps in Table IV may be resolved. Diiodotyrosine emerges with leucine and isoleucine, but can be differentiated on a butyl-benzyl alcohol chromatogram (2). Tryptophan coincides with phenylalanine in the solvent mixture referred to in Table IV, but can be determined with 0.1 N aqueous HCl (*cf.* Fig. 3). In acid hydrolysates of proteins the problem seldom arises, since tryptophan is usually decomposed during the hydrolytic process (2). Either α-amino-*n*-butyric acid or α-aminoadipic acid, if present, would appear as a new peak midway in the valley between tyrosine and proline. Cysteic acid has been found to give a clearly defined peak on the right side of the curve for glutamic acid plus alanine. Taurine is indistinguishable from threonine in this solvent, but moves ahead of glutamic acid in 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. Hydroxyproline travels at a rate similar to that of aspartic acid. Although hydroxyproline cannot be determined in this solvent mixture, its color yield is only 0.03 at 570 mμ, and unless present in unusually large quantities, it will not interfere with the estimation of aspartic acid. Citrulline and ethanolamine are slightly to the left and the right, respectively, of serine. The presence of either of these substances will be manifested by a broadening of the peak in the serine position. Glucosamine, if present, would appear as a peak midway between ammonia and arginine. Ornithine and hydroxylysine both coincide with cystine. With protein hydrolysates, therefore, the maximum possible amount of cystine present should be calculated from the total sulfur minus the methionine sulfur. If the amount of ninhydrin color in the cystine position exceeds that allowed by the calculation, the presence of additional components in the cystine range is indicated.

The fact that D-, L-, and DL-amino acids travel at the same rates on starch columns (2) has been checked in the present experiments with the L and DL forms of proline, glutamic acid, alanine, threonine, aspartic acid, and serine.

Behavior of Cysteine—When a freshly prepared solution of cysteine hydrochloride is added to a column with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl as the solvent, the amino acid is gradually oxidized to cystine and no cysteine peak is obtained in the effluent. No ninhydrin-positive material emerges from the column until after the shift of solvent to 2:1 *n*-propyl alcohol-0.5 N HCl. In the range of arginine a long flat zone begins and continues up to the position of cystine, where a definite peak occurs. The absorption maximum of the material in this broad zone is at 570 mμ, whereas the absorption maximum of cysteine is at 470 mμ (3). The acidity of the initial solvent is thus insufficient to maintain cysteine in the reduced state. When this amino acid is allowed to stand in the 1:2:1 solvent at atmospheric pressure, about 45 per cent of the cysteine is oxidized in 24 hours. The rate of oxidation on the column is probably accelerated by the increased amount of air in the solvent which enters the column under 15 cm. pressure.

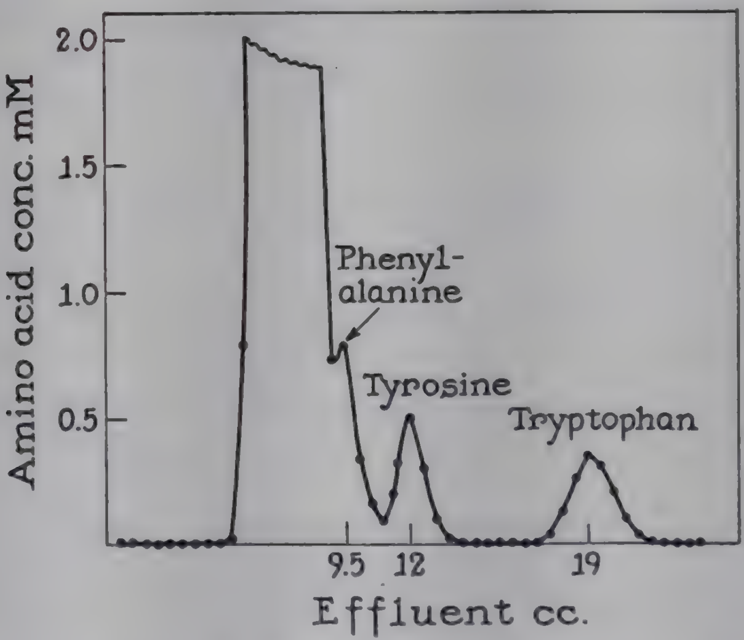

FIG. 3. Separation of tryptophan, with 0.1 N HCl as solvent, from a synthetic mixture containing eighteen components.

Cysteine, if present in a sample of amino acids applied to the column, would interfere with the determinations of the basic amino acids. In the chromatographic work with protein hydrolysates, however, the presence of cysteine has not, thus far, been detected (*cf.* (4)).

Cysteine is fairly stable in the more strongly acid solvent, 2:1 *n*-propyl alcohol-0.5 N HCl. If the column is run from the beginning with this solvent mixture, a cysteine peak (absorption maximum 470 mμ) is obtained near the position of threonine (*cf.* Fig. 4).

Separation of Tryptophan—The behavior of tryptophan on a column run with aqueous 0.1 N HCl has been referred to previously (1, 2). The column for this purpose is poured in butyl alcohol and washed with 1:1 *n*-propyl alcohol-0.5 N HCl as usual before being shifted to 0.1 N HCl. The curve obtained with the synthetic bovine serum albumin mixture to which tryptophan had been added is given in Fig. 3. The first peak contains most of the components of the mixture. Only the aromatic amino acids are appreciably retarded, and tryptophan emerges as a completely separated peak. The color yield of tryptophan in the neutralized 0.5 cc. samples has been 0.72 (3) and the recoveries from the chromatogram have been 100 ± 3 per cent.

Esterification in Acidic Solvents—The fact that the amino acids should not be allowed to stand in an acidic alcoholic solvent before the sample is placed on the column has been noted previously (2). Aspartic and glutamic acids are the only amino acids which have shown measurable esterification on the starch column during the course of the present experiments. The degree of ester formation is a function of the amount of water in the solvent mixture, the HCl concentration, the nature of the alcohols, and the time of contact. In the chromatograms with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl the recoveries of aspartic and glutamic acids have been 6 and 7 per cent low, respectively. The percentage loss is independent of the amounts of the amino acids present. If the column is developed from the beginning with 2:1 *n*-propyl alcohol-0.5 N HCl (*cf.* Fig. 4), the losses of these two amino acids are 10 and 20 per cent. On a column developed with *n*-butyl alcohol-17 per cent 0.57 N HCl (2), the recoveries are low by 20 and 30 per cent.

If the synthetic mixture of amino acids is allowed to stand for 1 week in the 1:2:1 solvent before the sample is placed on the column, two small additional peaks appear ahead of leucine plus isoleucine in Fig. 1. The yield of glutamic acid is about 20 per cent low and that of aspartic acid about 10 per cent low. All other components are quantitatively recovered. The small amount of esterification which occurs during the usual chromatographic experiment is not manifest in any way other than in the reduction of the yields of aspartic and glutamic acids. The esters, as they are continuously formed, move fairly rapidly through the column and doubtless contribute some ninhydrin color to all the effluent fractions preceding the glutamic and aspartic acid peaks. The quantity of ester is so small, however, and is distributed over so many fractions that the increase in ninhydrin color for any given fraction is almost imperceptible.

It has already been noted that in the mixture of secondary and tertiary alcohols used for the separation of glutamic acid and alanine (Fig. 2) esterification appears to be negligible.

Studies on Other Solvent Mixtures—In the chromatographic separation of the faster moving amino acids described in the previous communication (2), neutral water-immiscible organic solvents such as *n*-butyl alcohol and benzyl alcohol were used with columns 30 cm. in height. In order to elute some of the slower moving amino acids from such columns, inconveniently large effluent volumes are required. As the concentrations in the effluent become more dilute, the analytical accuracy is decreased. By the use of these same solvents with shorter columns (7.5 cm. in height), proline, alanine, and threonine can be eluted satisfactorily (1). The number of effective plates in a column, however, or the potential resolving power of the chromatogram is proportional to its length, and consequently it is preferable to use the longest column compatible with convenient laboratory operation. In order to attain satisfactory rates of travel for the slower moving amino acids on columns 30 cm. in length, a variety of solvent mixtures have been studied.

Neutral *n*-propyl alcohol-water mixtures were investigated on starch columns poured in butanol, washed to constant blank with the neutral solvent, and treated with 8-hydroxyquinoline (2). With 2:1 *n*-propyl alcohol-water, a curve was obtained which was similar to the first portion of Fig. 1, except that glutamic acid and aspartic acid were not present as discrete peaks but were spread out in a long low plateau extending from 60 to 100 cc. of the effluent. The other amino acid peaks emerged somewhat ahead of their positions in Fig. 1, threonine being at 51 cc. The last peak, which emerged at 71 cc., was composed of both serine and glycine. There also appeared, between proline and alanine, a large artifact peak which proved to result from ninhydrin-positive material eluted from the starch by the HCl in the amino acid sample. It was found that a small amount of either HCl or NaCl, when added to the top of the column, was capable of liberating material containing amino nitrogen, which moved down the column as a discrete zone and emerged as an irregular peak just ahead of the alanine position. The 2:1 *n*-propyl alcohol-water experiment provided a possible determination of proline, alanine, and threonine. The presence of the artifact peak and the unsatisfactory behavior of the acidic amino acids were marked disadvantages.

Glutamic acid and aspartic acid were obtained as normally sharp peaks in the alanine-threonine range when 0.25 N acetic acid was substituted for water in the 2:1 mixture with *n*-propyl alcohol. The artifact peak was still present, however, and there was overlapping of the components. In an attempt to eliminate the artifact, the starch column was treated with HCl and propyl alcohol, as described in the experimental section, until all ninhydrin-positive material had been eluted. The solvent was then changed to 2:1 *n*-propyl alcohol-water. When an amino acid mixture containing no HCl or NaCl was added to the column, the amino acid peaks were markedly retarded by the acid-washed starch. As an alternative procedure, a column was cleaned until ninhydrin-negative by using 2:1 *n*-propyl alcohol-0.1 N NaCl. The column was washed free of chloride ion with 2:1 *n*-propyl alcohol-water and the amino acid sample was added as usual. With the NaCl-washed starch the neutral amino acid peaks were sharp and there was no artifact zone. The peaks of the acidic amino acids, however, although they appeared in the proper range, were markedly broadened. As a result the curve was similar to Fig. 1, except that alanine, glutamic acid, threonine, and aspartic acid emerged as a group. Serine and glycine gave overlapping peaks at 67 and 71 cc. The inclusion of 0.5 N acetic acid or 0.5 N pyridine in the solvent did not improve the resolution in the acidic amino acid range. If acid-washed starch was suspended briefly in dilute NaOH and washed free of alkali, a product was obtained which behaved similarly to NaCl-washed starch.

Thus, the acidic amino acids have not yielded fully satisfactory results on starch columns developed with neutral unbuffered solvents. In addition, the properties of starch are such that both unwashed and NaCl-washed samples have a strong affinity for the basic amino acids. Even when only water is used as the solvent, the basic amino acids travel extremely slowly. Although the characteristics of neutral columns have thus not proved favorable for analytical work, it is possible that they may be useful in certain cases for preparative experiments. The effluent contains a minimum of carbohydrate impurities, whereas the effluent from columns run with acidic solvents is ninhydrin-negative but not carbohydrate-free. Although the columns prepared with acidic solvents retain their efficiency over periods of several weeks, starch is not fully stable under these conditions and some carbohydrate material is continuously passing into the effluent. The separation of amino nitrogen-containing constituents from carbohydrates in the effluent does not present major difficulties in some cases, but further work is required to facilitate the isolation of components from the effluent of columns run with acidic solvents.

In early experiments, attempts were made to achieve satisfactory rates of travel of amino acids on the column simply by varying the water content of propanol-water mixtures. It was found, however, that, although the amino acids emerged at greater effluent volumes as the amount of water in the solvent was decreased, this retardation was accompanied by a broadening and flattening of the peaks when the water content was reduced below about 30 per cent. Thus, 3:1 *n*-propyl alcohol-water gives a curve in which a peak emerging at a given effluent volume is slightly lower and broader than its counterpart in a 2:1 solvent. If the water content is reduced from 25 to 20 per cent, a comparison of the peaks emerging at the same effluent positions shows those in the 4:1 solvent to be about halved in height and doubled in width. A further reduction in the amount of water, to 12 per cent, causes the amino acids to emerge at a fairly steady low concentration level devoid of discrete peaks and valleys. Similar effects are noted with *n*-butyl alcohol when the water content is reduced below 15 per cent. Combinations of *n*-propyl and *n*-butyl alcohols, as used in the 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl solvent, permit mixtures to be employed which have water contents intermediate between 15 and 30 per cent without there being manifest any undesirable broadening of the peaks.

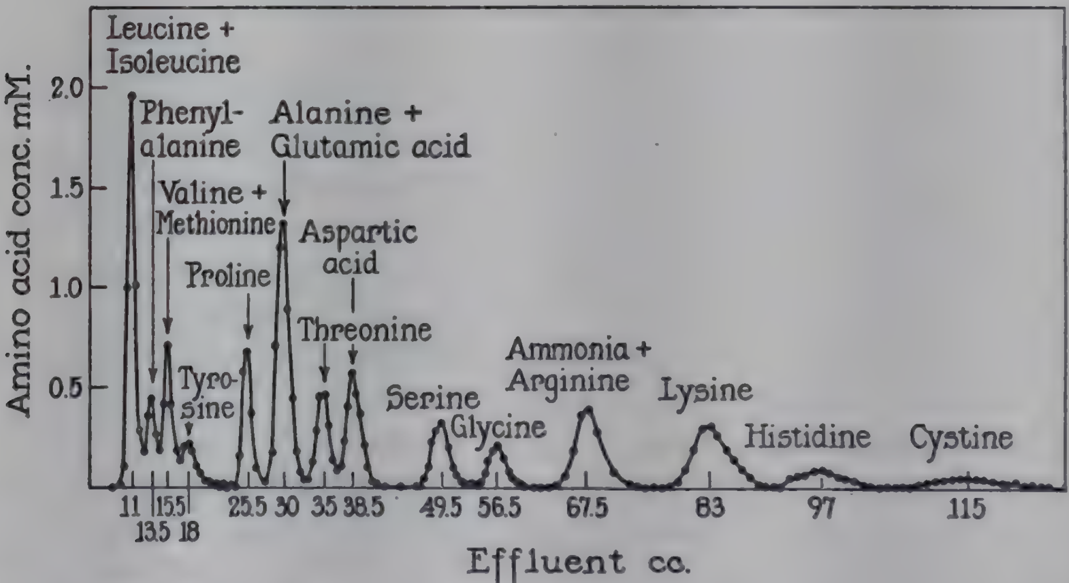

FIG. 4. Separation of amino acids on a chromatogram carried out with 2:1 *n*-propyl alcohol-0.5 N HCl.

It may prove desirable for some purposes to run the column from the beginning with 2:1 *n*-propyl alcohol-0.5 N HCl, instead of employing a solvent of lower water and HCl content for the first part of the curve. The results of such an experiment are shown in Fig. 4. The resolution of the faster moving amino acids is less satisfactory than in Fig. 1. The losses of glutamic and aspartic acids as a result of esterification are greater, as already noted. The fact that ammonia and arginine emerge together is a disadvantage. Nevertheless, the solvent may have some utility for screening work. A general picture of the composition of a mixture of amino acids is obtained in a 4 day experiment, instead of the 7 days required to obtain the results shown in Fig. 1.

Many experiments with acidic solvents other than the ones already described were carried out in an attempt to increase the resolution of the amino acids in the proline-aspartic acid range. Usually a preliminary experiment was performed with the synthetic serum albumin mixture. Inspection and integration of the curves were frequently sufficient to eliminate a given solvent from further consideration. Some of the solvents were investigated in greater detail with simpler mixtures of amino acids. The only combination found which would completely separate glutamic acid and alanine was 3:1 *tert*-butyl alcohol-0.1 N HCl. Because of its viscosity, this solvent gives excessively slow flow rates on the starch columns and has not been used routinely. The incorporation of 25 per cent *sec*-butyl alcohol in the mixture has given a satisfactory flow rate and reasonably good separation of the two amino acids. Various other mixtures of 0.1 N HCl with *sec*-butyl alcohol, *tert*-butyl alcohol, isobutyl alcohol, isopropyl alcohol, methyl cellosolve, and butyl cellosolve were tried. The mixtures did not offer any general advantages over the solvents referred to in Figs. 1 and 2.

In the present experiments, emphasis has been focused on solvents containing no non-volatile acids or salts which would tend to complicate the possible isolation of constituents from the effluent. A few chromatograms have been run with buffered solutions and with non-volatile acids. In 2:1 *n*-propyl alcohol-0.5 N H_3PO_4, the results were fairly similar to those shown in Fig. 4. In 2:1 *n*-propyl alcohol-0.5 N trichloroacetic acid, the basic amino acid peaks were advanced to positions on top of the components in the alanine-glycine range. No advantages in the proline-aspartic acid range were afforded by the use of 2:1 *n*-propyl alcohol-0.5 N monochloroacetic acid. With 2:1 *n*-propyl alcohol-0.2 N citric acid the peaks were markedly broadened and resolution was inferior.

When buffered solutions are used on starch columns, sharp peaks are obtained with both the acidic and basic amino acids. In 2:1 *n*-propyl alcohol-0.2 M citrate buffer, pH 5, the curve was similar to that in Fig. 1, except that glutamic acid and aspartic acid were shifted to the right. Glutamic acid emerged at a position on top of serine and glycine and was followed by the aspartic acid peak. The chromatogram was not continued to cover the basic amino acid range. When a citrate buffer of pH 4 was used, the basic amino acids were moved up to give an overlapping zone with glycine, serine, ammonia, and the acidic amino acids. In 3:2 *n*-propyl alcohol-0.08 M citrate buffer, pH 8, the relative rates of travel of the basic amino acids were further increased to give a heavily bunched group in the center section of the curve. Solvents that are much more alkaline than pH 8 cannot be used with starch. With 0.1 N NaOH, the starch at the top of the column swells and gelatinizes in the presence of the strong alkali.

Thus, both organic acids and the citrate buffers of pH 4 and 8 increase the rates of travel of the basic amino acids relative to the monoamino acids, thereby increasing the probability of overlaps in the chromatogram. The use of HCl possesses the advantage that minimum rates of travel for the basic amino acids are obtained, placing them in a region to the right of glycine.

SUMMARY

Chromatographic fractionation of amino acids on starch columns has been extended to include most of the common constituents of protein hydrolysates. The principal solvent mixture which has been used is 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl followed, after the emergence of aspartic acid, by 2:1 *n*-propyl alcohol-0.5 N HCl. In experiments with synthetic mixtures containing seventeen amino acids and ammonia, this combination of solvents yields in a single chromatogram a curve which includes all the components, with a few overlaps. For analytical work, about 2.5 mg. of the amino acid mixture are required per chromatogram. Integration of the curves has given quantitative recoveries for proline, threonine, aspartic acid, serine, glycine, ammonia, arginine, lysine, histidine, and cystine. Glutamic acid and alanine emerge together but can be resolved in a separate chromatogram with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. The six most rapidly moving components are partially resolved and have been separated, as previously reported, on columns run with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water for the determination of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. Thus, by the use of three starch columns it is possible to separate from one another all the eighteen components.

The average recoveries in duplicate or triplicate determinations have been 100 ± 3 per cent. The positions of emergence of some of the less commonly occurring amino acids and related compounds have been determined. Tryptophan, although not usually present in acid hydrolysates, presents a special case and can be determined on a column developed with aqueous 0.1 N HCl. If desired, a variety of other solvents, including neutral, acidic, and buffered solvent mixtures, can be used satisfactorily with starch columns.

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AMINO ACID COMPOSITION OF β-LACTOGLOBULIN AND BOVINE SERUM ALBUMIN

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The experiments described in this communication are concerned with the use of starch columns (1–3) for the chromatographic determination of the amino acid composition of protein hydrolysates. The techniques outlined in the preceding paper (3) have been employed to analyze hydrolysates of β-lactoglobulin and bovine serum albumin. In conjunction with the results of earlier chromatograms run with the butyl-benzyl alcohol solvent system (1), the analyses appear to have accounted for all the amino acids present in acid hydrolysates of these two proteins. Since numerous amino acid determinations have already been reported for both β-lactoglobulin and bovine serum albumin, an opportunity is afforded for comparison of the chromatographic data with the results obtained by other methods.

The experimental procedure used for the chromatographic analyses was identical with that employed in the preceding studies with synthetic mixtures of amino acids (3). The sample of β-lactoglobulin used in this work was prepared by Dr. G. Haugaard according to the method of Palmer (4, 5), and was one of the samples recently analyzed by Brand and coworkers (6). The bovine serum albumin, prepared according to the method of Cohn *et al.* (7), was obtained through the kind cooperation of Dr. Erwin Brand, and was the same preparation (Armour, Lot 18) analyzed in his laboratory. Hydrolysis was performed in the manner already outlined (1), with 200 volumes of 6 N HCl twice distilled in glass. For convenience in manipulation, 250 to 500 mg. of protein were hydrolyzed. Since the chromatographic analyses require only 2.5 mg. per experiment, the procedure for hydrolysis and for addition of the sample to the column can be scaled down, if desired, to permit the series of chromatograms to be completed with 25 to 50 mg. of protein.

Analyses of Hydrolysates of β-Lactoglobulin

A hydrolysate of β-lactoglobulin was chromatographed with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl as solvents. The resulting effluent concentration curve, given in Fig. 1, is similar in all respects to the curve obtained in experiments with a synthetic mixture containing seventeen amino acids and ammonium chloride (3). The general pattern and the approximate positions of the peaks are the same in the two curves. There are no peaks in Fig. 1 which cannot be ascribed to the common amino acids. No evidence has been obtained, therefore, for the existence in β-lactoglobulin hydrolysates of unsuspected amino nitrogen-containing constituents. In order to confirm the positions assigned to the peaks in Fig. 1, threonine, serine, and histidine were added to the hydrolysate. The designated peaks rose without loss of symmetry. Since β-lactoglobulin is a protein, the composition of which has already been explored rather fully by a variety of methods, the identification of the peaks in the chromatogram can be made with a relatively high degree of certainty (1, 3). Characterization of the components of the peaks by isolation of the amino acids from the effluent has not been attempted in these analytical experiments.

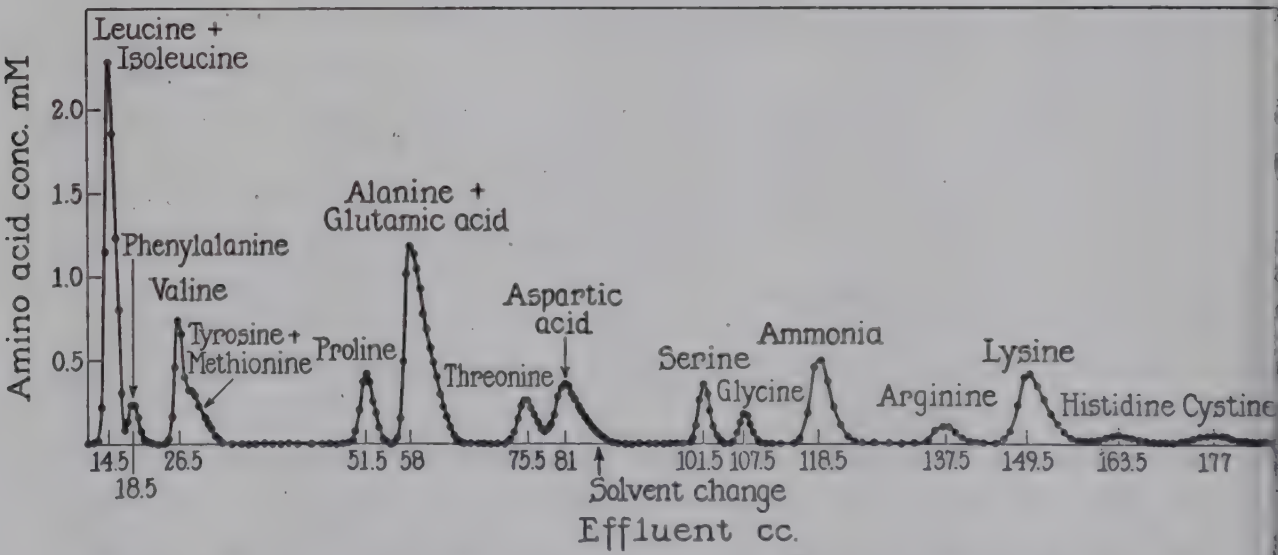

FIG. 1. Chromatographic fractionation of a hydrolysate of β-lactoglobulin. Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl. Column dimensions, 0.9 × 30 cm. Sample, an amount of hydrolysate corresponding to about 2.5 mg. of protein.

Integration of Effluent Curves—The quantitative data yielded by the experiment shown in Fig. 1, and replicates thereof, are given in Table I. The total nitrogen recoveries of 98.9 and 99.7 per cent indicate that the chromatogram has almost completely accounted for the nitrogenous components of the hydrolysate. There are several approximations involved, however, in this calculation. As already mentioned (3), the peak for valine plus methionine plus tyrosine yields only an approximate value for the per cent of the total nitrogen of the protein attributable to these combined amino acids. The peak is integrated by using a color yield of 1.00, whereas the respective color yields for the individual components are 1.01, 1.00, and 0.86. The recoveries obtained for the first six fast moving components in Fig. 1 are useful in the preparation of a nitrogen balance sheet for the chromatogram, but are replaced by the results from a butyl-benzyl alcohol column in later calculations. The glutamic acid plus alanine integration also yields a slightly low figure, since a correction for losses of glutamic acid as a result of esterification is not included. Tryptophan is not present in the acid hydrolysate, as evidenced by the absence of ninhydrin-positive material in the valley between phenylalanine and leucine in the earlier butyl-benzyl alcohol chromatograms (1). Since tryptophan represents 1.71 per cent of the protein nitrogen, as determined by Brand and coworkers (6) who published the first approximately complete analysis of β-lactoglobulin, the theoretical recovery for Table I should be 98.3 per cent. For some purposes, such as comparative studies on different protein preparations, the type of data afforded by Table I may be adequate without the performance of additional chromatograms. Quantitative figures are obtained for ten of the individual components, and an approximate total nitrogen recovery may be derived from the single chromatogram.

TABLE I

Chromatographic Analyses of Hydrolysates of β-Lactoglobulin

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl (*cf*. Fig. 1).

Constituent	Chromatogram 472		Chromatogram 491		Chromatogram 486*
	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein
Leucine-isoleucine	22.6	15.47	22.4	15.34	
Phenylalanine	3.85	2.09	3.89	2.12	
Valine-methionine-tyrosine		8.38 *ca.*		8.30 *ca.*	
Proline	5.29	4.13	5.16	4.03	4.96
Glutamic acid-alanine		18.60 *ca.*		19.35 *ca.*	
Threonine	4.85	3.65	4.75	3.58	4.42
Aspartic acid	11.46	7.74	11.64	7.86	11.47
Serine	3.56	3.04	3.76	3.21	3.37
Glycine	1.35	1.62	1.48	1.77	1.34
Ammonia	1.44	7.61	1.47	7.76	1.45
Arginine	3.07	6.34	2.74	5.66	2.91
Lysine	12.25	15.05	12.70	15.60	12.80
Histidine	1.49	2.59	1.61	2.80	1.78
Cystine	3.52	2.63	3.15	2.35	3.09
Total nitrogen recovery		98.9		99.7	

* A 40 cc. fore fraction was collected and the column placed on the fraction collector before the emergence of the proline peak.

The hydrolysates have given a negative nitroprusside test. In addition, there was no evidence of interference from cysteine in the valleys on either side of the histidine peak. Apparently any cysteine originally present is oxidized during the preparation of the hydrolysate for analysis.

In the present experiments the data from Table I have been combined with the results from additional columns to give a more complete picture of the composition of the hydrolysate. Glutamic acid and alanine have been determined with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl (3). A fore fraction was collected and the chromatogram placed on the fraction collector prior to the emergence of proline. Duplicate determinations gave values of 19.30 and 18.85 per cent for glutamic acid, and 7.10 and 7.08 per cent for alanine. The results obtained in the experiments with *n*-butyl alcohol-benzyl alcohol columns have already been reported (1).

Composition of β-Lactoglobulin—The amino acid composition of β-lactoglobulin indicated in Table II is based in major part upon the analytical results from the three types of chromatograms discussed above. From the standpoint of protein composition, data of the kind given in Table II are always subject to uncertainties arising from possible decomposition of various amino acids during hydrolysis. Significant amounts of threonine and serine, for example, are known to decompose during acid hydrolysis, with the formation of ammonia. In the present work, the values of 4.67 and 3.56 for threonine and serine, obtained from the hydrolysate, have been divided by 0.95 and 0.90, respectively, in accordance with the estimates of Rees (17) for the decomposition of these amino acids during a 20 hour period of acid hydrolysis. Although the hydrolytic conditions employed in the present experiments differ from those used by Rees, the parallelism between his results and the present figures indicates that the same correction factors are probably applicable in both cases. For example, Rees obtained uncorrected values of 4.84 and 3.64 per cent for threonine and serine in hydrolysates of β-lactoglobulin. He found the total ammonia content of the hydrolysate to be 1.49 per cent. The corresponding chromatographic value is 1.45 per cent (Table I).

A maximum possible value for the amide ammonia of the protein can be obtained from the chromatographic data by subtracting from the total ammonia content of the hydrolysate the amount of ammonia estimated to be formed by the decomposition of threonine and serine. The resulting figure, 1.35 per cent of ammonia, is slightly higher than the amide ammonia value of 1.31 per cent as determined by Warner and Cannan (20), Brand *et al.* (6), and by Rees (17).

Essential data in Table II derived from other sources include the photometric value of Brand *et al.* (6) for tryptophan and the sulfur partition results obtained by the same authors. The independent value for me-

TABLE II

Amino Acid Composition of β-Lactoglobulin

The values for phenylalanine, leucine, isoleucine, tyrosine, and valine are from chromatograms carried out with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water (1). Glutamic acid and alanine were determined with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. The remaining chromatographic values are the average figures from Table I. The nitrogen content of the protein was 15.6 per cent, on an ash- and moisture-free basis.

Constituent	Gm. amino acid per 100 gm. protein	Gm. amino acid residue per 100 gm. protein	N as per cent of protein N	Literature values, gm. amino acid per 100 gm. protein
Phenylalanine	3.78	3.37	2.06	3.54 (6), 4.3 (8), 4.2 (9)
Leucine	15.50	13.38	10.61	15.7 (7), 15.4 (10), 15.9 (11)
Isoleucine	5.86	5.05	4.01	8.4 (6), 7.0 (8), 6.1 (12)
Methionine	3.22[*]	2.84	1.94	
Tyrosine	3.64	3.28	1.81	3.78 (6), 3.6 (13)
Valine	5.62	4.76	4.31	5.8 (6), 5.5 (8), 5.8 (9)
Proline	5.14	4.33	4.01	4.1 (6), 5.5 (9), 4.84 (14)
Glutamic acid	19.08	16.75	11.64	19.5 (6), 19.1 (15), 21.51 (16)
Aspartic "	11.52	9.98	7.80	11.4 (6), 11.2 (15), 9.9 (16)
Alanine	7.09	5.67	7.15	6.2 (6), 6.1 (9), 7.05 (14)
Threonine	4.92[†]	4.18	3.71	5.85 (6), 5.11 (17)
Serine	3.96[†]	3.28	3.39	5.0 (6), 4.07 (17)
Glycine	1.39	1.06	1.66	1.4 (6), 1.5 (15), 1.56 (14)
Arginine	2.91	2.61	6.00	2.87 (6), 2.89 (16), 2.91 (18)
Lysine	12.58	11.02	15.45	11.4 (6), 11.4 (15), 10.55 (19)
Histidine	1.63	1.44	2.83	1.58 (6), 1.50 (8), 1.55 (16)
Cystine + cysteine	3.40[‡]	2.89	2.54	
Tryptophan	1.94[§]	1.77	1.71	
Amide-NH_3	1.31[‖]		6.93	1.31 (6), 1.30 (17)
Total		97.7	99.6	

[*] The value for methionine is that determined by Brand *et al.* (6). An independent check on this figure was not obtained with the butyl-benzyl alcohol chromatograms in this case, since the experiments were carried out before the introduction of thiodiglycol as an antioxidant.

[†] The average threonine and serine values of 4.67 and 3.56 from Table I have been divided by 0.95 and 0.90, respectively, in accordance with the estimates of Rees (17) for decomposition of these amino acids during hydrolysis.

[‡] The cystine + cysteine value is that determined by Brand *et al.* (6). The chromatograms gave an average value of 3.25.

[§] The tryptophan value is that determined by Brand *et al.* (6).

[‖] The amide-NH_3 value is that determined by Warner and Cannan (20). From the chromatographic data a maximum amide-NH_3 value of 1.35 can be calculated.

thionine is necessary in this case, since the butyl-benzyl alcohol chromatograms were performed before the incorporation of thiodiglycol in the solvent as an antioxidant (1). Brand *et al.* (6) obtained a value of 3.40 per cent for cysteine plus cystine, which, in conjunction with the methionine content, accounted for 99.6 per cent of the sulfur of the protein. The average chromatographic value in Table I is 3.25 per cent. It should be noted that the hydrolytic conditions employed for the chromatographic experiments were not the same as those recommended as optimum for cysteine and cystine determinations by Brand and Kassell (21). Values for cystine in the present hydrolysates have run from 5 to 10 per cent below the accepted figures for cysteine plus cystine. The quantitative recovery of cystine from synthetic mixtures (3) indicates that the chromatogram reflects the true cystine content of the hydrolysate, and that the values are low as a result of some decomposition of cysteine or cystine during the preparation of the hydrolysate. In the present experiments, an attempt has been made to obtain as much information as possible from an HCl hydrolysate. Adequate chromatographic values for the special case of cystine would require studies on hydrolysates prepared by alternative procedures.

The fact that the calculated cystine sulfur from the chromatogram is slightly less than enough to account, together with methionine, for the total sulfur of the protein leaves no room for the presence of ornithine or hydroxylysine as possible overlaps in the cystine range (3).

The total nitrogen recovery of 99.6 per cent in Table II rests primarily on the chromatographic data. The weight recovery is 97.7 per cent. For many purposes this is a satisfactory balance sheet for the protein. The 2 per cent unaccounted for on a weight basis is a discrepancy which may arise from one or more of several causes. There may be some decomposition of amino acids other than those for which corrections have been made. Some of the amino acid analyses may be in error in such a manner as to compensate on a nitrogen basis but not on a weight basis. There may be amino acid constituents present which have not been determined by the chromatograms. Hydroxyproline, for example, would fall under the aspartic acid peak, and would not be detected. Negative colorimetric tests for this amino acid were obtained by Brand *et al.* (6). Recently, Keston, Udenfriend, and Cannan[1] have demonstrated by their isotopic derivative method, using radioactive *p*-iodophenyl sulfonyl chloride, that hydroxyproline is completely absent (<0.05 per cent) in β-lactoglobulin. It is also possible that the sample of protein contains small amounts of non-nitrogenous constituents or impurities in addition to the ash and moisture. The latter have been corrected for in the assignment of a nitrogen content of 15.6 per cent to the protein. In the present state of accuracy of the chromatographic methods, however, it is not possible to base any conclusions on the observed small difference between the weight and nitrogen recoveries.

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[1] Keston, A. S., Udenfriend, S., and Cannan, R. K., personal communication.

Comparison of Chromatographic Results with Other Values—Analytical results obtained by other methods are included in Table II. A more complete summary of literature values has been given by Brand (22). On synthetic mixtures of amino acids the chromatograms have yielded recoveries of 100 ± 3 per cent for individual components (1, 3). The microbiological assays, and some of the other methods, have a potential error at least this large. Thus, for purposes of comparison, conformity to within 5 per cent has been considered as reasonable agreement.

For leucine, tyrosine, valine, arginine, and histidine, the several literature values in Table II are all in agreement with the chromatographic results. Glycine also falls in this group if the variation from 1.39 to 1.56 per cent is considered satisfactory for an amino acid present to so small an extent. The threonine and serine values are in excellent agreement with those of Rees (17) obtained by periodate oxidation, as already mentioned, but are about 20 per cent below the results of Brand *et al.* (6). The glutamic and aspartic acid figures are both checks with the isotope dilution values of Foster (15) and the microbiological results of Brand *et al.* (6). They are not in line with the analyses of Chibnall, Rees, and Williams (16). The isoleucine result has been discussed previously (1) and is in agreement with the most recent microbiological determination (12). For phenylalanine, the two microbiological values (6, 8) are more than 5 per cent above and below the chromatographic value from starch columns. The figure obtained by Tristram (9), who employed chromatography of the acetylated amino acids on silica gel, is also higher than the present phenylalanine determination.

Alanine has previously been a difficult amino acid to determine. The present value is higher than the microbiological figure (6) or that of Tristram (9), but is in agreement with the recent results of Keston, Udenfriend, and Cannan (14), obtained by the isotopic derivative method. The proline content is 20 per cent above the microbiological value of Brand *et al.* (6), but in fairly good agreement with the isotopic derivative data (14).

The value for lysine given in Table II is 10 per cent higher than the microbiological (6) and isotope dilution (15) figures. References to additional lysine determinations, all of which fall between 10.5 and 11.4 per cent, are reviewed by Brand (22). The chromatographic value in this instance may be in error. For bovine serum albumin, discussed later, the chromatographic determination of lysine is in good agreement with the isotope dilution data. In the β-lactoglobulin hydrolysate, there may be a small amount of some component other than lysine traveling in the same range on the column, although none of the additional substances studied to date (3) fall in this category. It is also possible that the chromatographic figure may be correct, if there is some racemization of lysine during hydrolysis. It has been shown previously (3) that the sum of the D and L isomers in the hydrolysate is determined by the chromatographic method. As usually employed, the microbiological (6), isotope dilution (15), and lysine decarboxylase (23) methods permit the determination of only the L isomer.

A calculation of a minimum molecular weight for the protein from analytical figures has been made by Brand *et al.* (6), by adjusting the molar ratios to integers within 3 per cent. For the amino acids present in the smallest amounts in the protein, the attainment of this degree of accuracy by the chromatographic methods, at least, cannot be assured. For purposes of molecular weight calculations, an absolute accuracy of 5 per cent is about as close a limit as can be placed on the smaller figures in Table II. For example, the deviations from the mean in the triplicate analyses for arginine and histidine in Table I emphasize the need for caution in the mathematical interpretation of the results. The determination of histidine (1.63 per cent) to within 5 per cent of its value is equivalent to determining about 0.1 per cent of the total weight of the protein. This is about the limit of accuracy of the chromatographic procedure. The value of any detailed mathematical treatment of the analytical figures in this case is subject to the further limitation that β-lactoglobulin, although it is a protein which can be prepared in a fairly reproducible form, may not be homogeneous (*cf.* (24, 25)).

Analyses of Hydrolysates of Bovine Serum Albumin

The effluent concentration curve obtained upon chromatography of a hydrolysate of bovine serum albumin is given in Fig. 2. The curve parallels that obtained with the synthetic mixture (3) in all respects. No new peaks are present. When threonine, serine, and histidine were added to a sample of the hydrolysate, the designated peaks rose without loss of symmetry, and the added amino acids were recovered in yields of 105, 99, and 99 per cent, respectively.

Integration of Effluent Curves—The quantitative data obtained from the experiment shown in Fig. 2, and replicates thereof, are given in Table III. As was true in the case of β-lactoglobulin, the nitrogen of the hydrolysate has been almost completely accounted for in each experiment. The tryptophan content of bovine serum albumin, according to the data of Brand *et al.* (26, 22), corresponds to only 0.50 per cent of the total nitrogen of the protein, and the theoretical recovery for Table III is thus 99.5 per cent. The observed recovery is subject to the same approximations which were discussed for β-lactoglobulin.

Duplicate chromatograms with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl gave values of 16.7 and 16.3 per cent for glutamic acid and 6.32 and 6.17 per cent for alanine.

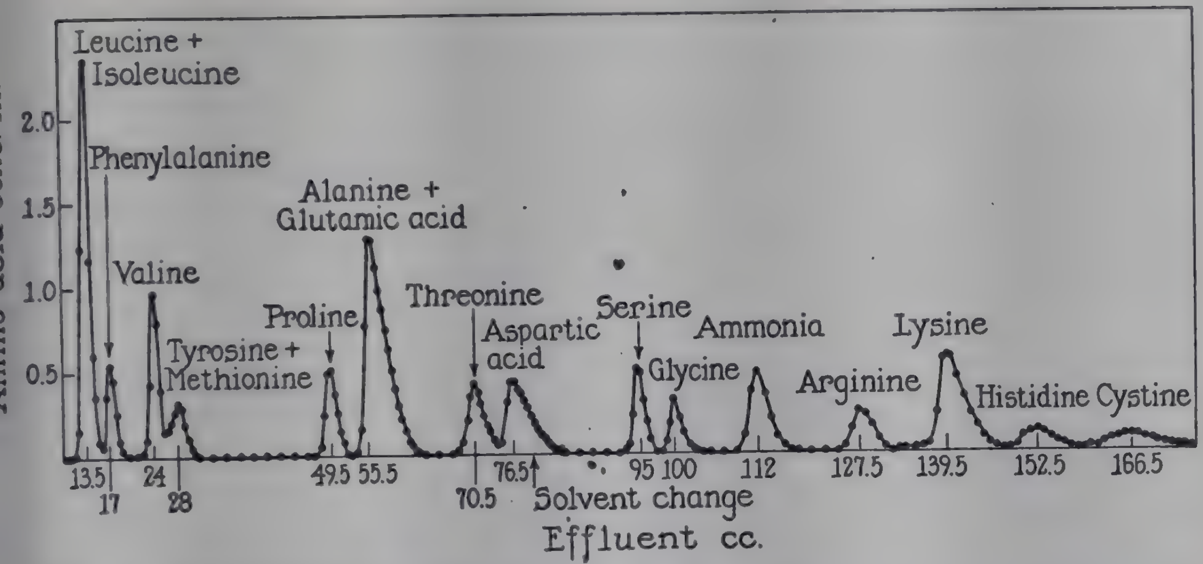

FIG. 2. Chromatographic fractionation of a hydrolysate of bovine serum albumin. Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl. Column dimensions, 0.9 × 30 cm. Sample, an amount of hydrolysate corresponding to about 2.5 mg. of protein.

TABLE III

Chromatographic Analyses of Hydrolysates of Bovine Serum Albumin

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl (*cf.* Fig. 2).

Constituent	Chromatogram 480		Chromatogram 481		Chromatogram 488	
	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein	N as per cent of protein N
Leucine-isoleucine	14.4	9.58	14.75	9.80	14.3	9.51
Phenylalanine	6.32	3.34	6.44	3.40	6.52	3.44
Valine-methionine-tyrosine		7.42 *ca.*		7.37 *ca.*		7.20 *ca.*
Proline	4.65	3.52	4.75	3.60	4.85	3.67
Glutamic acid-alanine		14.90 *ca.*		15.25 *ca.*		15.40 *ca.*
Threonine	5.44	3.98	5.72	4.19	5.50	4.05
Aspartic acid	10.91	7.15	10.86	7.12	10.96	7.19
Serine	3.91	3.24	3.76	3.12	3.77	3.13
Glycine	1.85	2.15	1.75	2.03	1.85	2.15
Ammonia	1.03	5.28	1.08	5.54	1.06	5.44
Arginine	5.96	11.92	6.03	12.07	5.72	11.45
Lysine	12.70	15.15	12.62	15.05	13.15	15.68
Histidine	4.29	7.24	3.67	6.18	4.04	6.82
Cystine	5.83	4.23	5.81	4.22	6.08	4.41
Total nitrogen recovery		99.1		98.9		99.5

TABLE IV

Amino Acid Composition of Bovine Serum Albumin

The values for phenylalanine, leucine, isoleucine, tyrosine, and valine are from chromatograms carried out with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water containing 0.5 per cent thiodiglycol (1). Glutamic acid and alanine were determined with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. The remaining chromatographic values are the average figures from Table II. The nitrogen content of the protein was 16.07 per cent, on an ash- and moisture-free basis.

Constituent	Gm. amino acid per 100 gm. protein	Gm. amino acid residue per 100 gm. protein	N as per cent of protein N	Literature values, gm. amino acid per 100 gm. protein
Phenylalanine	6.59	5.87	3.48	6.1 (22), 6.48 (27), 6.05 (28)
Leucine	12.27	10.58	8.17	13.7 (22, 26), 11.8 (27), 13.2 (28)
Isoleucine	2.61	2.25	1.74	2.9 (22), 2.97 (27), 2.7 (28)
Methionine	0.81*	0.71	0.47	0.86 (27), 0.80 (28)
Tyrosine	5.06	4.56	2.44	5.5 (22, 29), 5.2 (30), 5.3 (13)
Valine	5.92	5.01	4.41	6.5 (22), 5.4 (30), 6.6 (27)
Proline	4.75	4.00	3.60	5.6 (22), 5.1 (27), 5.5 (28)
Glutamic acid	16.50	14.49	9.78	16.9 (22), 16.6 (27), 16.95 (29)
Aspartic "	10.91	9.44	7.15	10.6 (22), 11.1 (27), 10.25 (29)
Alanine	6.25	4.99	6.12	
Threonine	5.83†	4.95	4.27	6.5 (22, 26), 6.2 (27), 6.3 (30)
Serine	4.23†	3.51	3.52	4.5 (22, 26), 4.9 (28)
Glycine	1.82	1.38	2.11	1.9 (22), 1.96 (29), 2.0 (28)
Arginine	5.90	5.29	11.80	6.2 (22, 26), 5.9 (27), 6.1 (30)
Lysine	12.82	11.25	15.30	12.4 (22), 12.4 (29), 12.3 (30)
Histidine	4.00	3.54	6.75	3.8 (22, 26), 3.35 (31), 4.1 (30)
Cystine + cysteine	6.52‡	5.54	4.73	
Tryptophan	0.58§	0.53	0.50	
Amide-NH_3	0.95‖		4.87	1.05 (22, 26)
Total		97.9	101.2	

* The value for methionine is that determined by Brand *et al.* (22, 26). The chromatograms gave a figure of about 0.92 (1).

† The average threonine and serine values of 5.55 and 3.81 from Table III have been divided by 0.95 and 0.90, respectively, in accordance with the estimates of Rees (17) for decomposition of these amino acids during hydrolysis.

‡ The cystine + cysteine value is that determined by Brand *et al.* (22, 26). The chromatograms gave an average value of 5.91.

§ The tryptophan value is that determined by Brand *et al.* (22, 26).

‖ This figure is a maximum value for amide-NH_3 calculated from the total NH_3 of the hydrolysate corrected for the approximate amount of NH_3 formed on the decomposition of serine and threonine.

Composition of Bovine Serum Albumin—The data on bovine serum albumin have been summarized in Table IV. The chromatograms gave a figure of 0.92 per cent for methionine (1) which is close to the value of 0.81

per cent as determined by Brand *et al.* (26). The same authors obtained a figure of 6.52 per cent for cysteine plus cystine which, in conjunction with the methionine content, accounted for 99.0 per cent of the sulfur of the protein. The average chromatographic value for cystine in the hydrolysate is 10 per cent below the above figure.

The total ammonia in the hydrolysate of bovine serum albumin (Table III) averages 1.06 per cent. Corrected for 0.068 per cent ammonia formed by the decomposition of serine and 0.040 per cent from threonine, the maximum possible figure for the amide ammonia of the protein becomes 0.95 per cent. This result, uncorrected for additional ammonia that might be formed as a result of the decomposition of other amino acids, has been used in Table IV. The amide ammonia determination of Brand (22) on bovine serum albumin gives the apparently high figure of 1.05 per cent.

The total nitrogen recovery in Table IV is 101.2 per cent. The weight recovery is 97.9 per cent. Possible causes for the discrepancy between the weight and nitrogen recoveries have already been mentioned.

Comparison of Chromatographic Results with Other Values—The results for the first six amino acids in Table IV have already been discussed (1). Additional values in Table IV include microbiological assays reported by Henderson and Snell (27), Gunness, Dwyer, and Stokes (13), Hier, Graham, Friedes, and Klein (30), and Velick and Ronzoni (28). As in the case of β-lactoglobulin, hydroxyproline has been shown to be absent (<0.006 per cent) in bovine serum albumin by Keston, Udenfriend, and Cannan.[1] For glutamic acid, arginine, and lysine, several values from the literature and the chromatographic results are all in agreement. The glycine figure, considering the small amount present, checks with the isotope dilution value of Shemin (29) and the microbiological assays (26, 28). The chromatographic value for aspartic acid is in agreement with the microbiological results (26, 27) and within 6 per cent of the isotope dilution value (29). The figure for proline is 5 to 15 per cent below the microbiological determinations (26–28). The values for threonine and serine are also lower than the earlier results. The chromatographic figure for histidine is in agreement with the results of the microbiological (30) and photometric (26) methods, but is higher than the value obtained by the isolation method of Vickery and Winternitz (31). Alanine has not previously been determined in bovine serum albumin.

SUMMARY

Chromatography of amino acids on starch columns has been applied to the determination of the composition of β-lactoglobulin and bovine serum albumin. A single chromatogram run with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl distributes the amino acids to give an effluent curve which yields quantitative values for ten of the components of the hydro-

lysate. When the integrations of the overlapping peaks were included, a nitrogen distribution was obtained in each case which accounted for 100 ± 2 per cent of the total protein nitrogen. A combination of chromatograms run with three solvent systems is required for quantitative estimation of essentially all the components of the acid hydrolysates. About 2.5 mg. of protein are used per chromatogram. The complete series can be carried out in triplicate with a hydrolysate prepared from 25 to 50 mg. of protein.

To estimate the composition of the protein from data obtained on the hydrolysate, the values for serine and threonine must be corrected for the decomposition undergone by these amino acids during hydrolysis. Values for tryptophan and cysteine plus cystine determined by other methods are also required. The final tabulation of the results on β-lactoglobulin and bovine serum albumin has given total nitrogen recoveries of 99.6 and 101.2 per cent respectively, and weight recoveries of 97.7 and 97.9 per cent.

The individual amino acid analyses have been compared with values previously obtained by chemical, microbiological, and isotopic methods.

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FACTORS AFFECTING THE RATE OF GROWTH OF LACTOBACILLUS CASEI

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It has been recognized for some time that *Lactobacillus casei* develops slowly in completely synthetic or semisynthetic media which contain all of the absolutely essential vitamins and amino acids. Usually little or no growth appears during the first 16 to 24 hours of incubation. Under some conditions the addition of asparagine, glutamine, glutamic acid (1), or an unknown factor or factors in peptone (2), or a combination of glutamine, *p*-aminobenzoic acid, and pyridoxal (3) will speed up the rate of growth of *L. casei*. Trypsinized preparations of casein and other purified proteins, the active principle of which has been named strepogenin (4, 5), also will reduce the lag phase of *L. casei*. Certain synthetic peptides of glutamic acid, for example serylglycylglutamic acid, have some strepogenin activity (6). Also, evidence has been presented that for optimum growth of *L. casei* during the first 16 hours of incubation glutathione and a factor associated with animal products, as well as strepogenin, must be supplied to the organism (7). The present paper reports some additional observations on the relation of strepogenin and certain other factors to the rate of growth of *L. casei*. The term strepogenin is used to describe the activity of trypsinized casein and that of various purified proteins and natural materials when compared to trypsinized casein as the standard.

Methods

Experiments designed to investigate *rate phenomena* are technically difficult to carry out with consistent results, since apparently minor variations in the inoculum culture medium and technique may profoundly influence the rate of growth (7). In order to obtain reasonably consistent results it is necessary to use carefully standardized procedures. Therefore, experimental details are described more fully than would normally be necessary.

Stab cultures of *Lactobacillus casei* were carried in a medium consisting of peptone 0.5 per cent, glucose 1.0 per cent, agar 1.5 per cent, and 0.5 ml. each of Salts A and B per 100 ml. of medium (8). Each week a subculture was made from a stab culture into 5 ml. of broth of the same composition contained in a 15 × 125 mm. tube. The subculture was incubated at 37° for 24 hours. Subsequently, broth to broth transfers were made daily for the remainder of the week. To prepare inoculum, a 24 hour broth culture was centrifuged, and the sedimented cells were washed twice with 5 ml. of sterile distilled water and resuspended in 10 ml. of water. This suspension was diluted with water to give a reading of approximately 95 on the galvanometer of the Evelyn photoelectric colorimeter fitted with a 520 mμ filter.

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The medium listed in Table I was used for the experiments. Materials to be tested, after appropriate treatment and dilution, were placed in chromic acid-cleaned colorimeter tubes and made up to a volume of 5 ml. with water. 5 ml. of medium were added to each tube, and the tubes were closed with plugs of non-absorbent cotton and autoclaved for 11 minutes at 115°. Higher sterilization temperatures and, to a lesser extent, longer periods of sterilization produced a brown discoloration of the medium which interfered with subsequent turbidimetric readings.

TABLE I

Basal Medium

Acid-hydrolyzed casein	3400 *mg.*	Biotin	0.1 γ
DL-Tryptophan	100 "	Folic acid	2.5 "
L-Cystine	50 "	$MnCl_2\cdot 4H_2O$	2.5 *mg.*
Glucose	10 *gm.*	L-Asparagine	125.0 "
Na acetate (anhydrous)	6 "	Salts A	
Adenine	2.5 *mg.*	K_2HPO_4	250.0 "
Guanine	2.5 "	KH_2PO_4	250.0 "
Uracil	2.5 "	Salts B	
Pantothenic acid	100 γ	$MgSO_4\cdot 7H_2O$	100.0 "
Riboflavin	100 "	NaCl	5.0 "
Thiamine HCl	100 "	$FeSO_4\cdot 7H_2O$	5.0 "
Nicotinic acid	100 "	$MnSO_4\cdot 4H_2O$	5.0 "
Pyridoxamine	200 "	Adjust to pH 6.8	
p-Aminobenzoic acid	20 "	Add distilled H_2O to 250.0 ml.	

After sterilization, each tube was inoculated with 1 drop of the inoculum suspension described above. It is necessary to use pipettes which will deliver drops of uniform size. 1 ml. Exax pipettes graduated in 0.01 ml. were satisfactory for delivery of the inoculum. Care was taken to allow the drop of inoculum to fall directly into the medium rather than on the walls of the tube. The inoculated tubes were incubated at 37° for 17 hours prior to determining their turbidities, or until a control tube containing 3 mg. of trypsinized casein gave a galvanometer deflection of 40 to 50 in the Evelyn colorimeter with a 520 mμ filter. Additional details of procedure will be mentioned later.

Purified proteins and natural materials were trypsinized according to the procedure of Sprince and Woolley (5). Difco trypsin 1:110 was used. The trypsinized casein standard was prepared from S. M. A. vitamin-free casein. To prepare standard curves of the trypsinized casein, the required amount of the lyophilized material was dissolved in water to give a concentration of 1 mg. per ml. This solution could be stored in the refrigerator under toluene for at least 2 weeks without loss of activity.

The hydrolyzed casein used in the basal medium was a sulfuric acid hydrolysate of S. M. A. vitamin-free casein from which the sulfate was removed with barium hydroxide.

EXPERIMENTAL

Inoculum—The rate of growth of *Lactobacillus casei* under the conditions outlined above is markedly increased by strepogenin and is proportional to strepogenin concentration, generally, within the range of 0 to 1 mg. of casein (Fig. 1). With increase in incubation beyond 16 hours, growth increases in all tubes, including the one with no strepogenin; at 42 hours the stimulatory effect of strepogenin is hardly evident.

Growth response of *Lactobacillus casei* to strepogenin is decreased somewhat if the cells for the inoculum are taken from a 2 day-old broth culture in place of the customary 1 day culture. Cells from 3 day-old broth cultures are only slightly stimulated by strepogenin at 16 hours incubation. Also, growth is proportional to the number of cells in the inoculum. A decrease in the number of cells to one-tenth that normally used decreases the response to strepogenin markedly, whereas a 10-fold increase produces much greater growth of *L. casei* in all tubes. The resulting curves resemble closely those obtained by varying the time of incubation (Fig. 1). Inocula grown in broth enriched with 0.25 mg. of L-asparagine per ml. and in broth in which Wilson's solubilized liver fraction L was substituted for the peptone were stimulated by strepogenin to the same degree as cells taken from the usual peptone medium.

Activity of Purified Proteins and Natural Materials—Sprince and Woolley have used solubilized liver extract (Wilson's fraction L) as the standard in measuring the strepogenin activity of trypsinized proteins (4, 5). Comparison of solubilized liver with trypsinized casein indicates that, within the range of 0 to 1 mg. of each, casein is about 4 times as potent as liver extract (Fig. 2). However, if both substances are increased to above 3 mg., there is a change in relative potency. The casein growth curve decreases markedly in slope at the 3 mg. level, whereas that of liver extract does not decrease until the 10 mg. level is reached. Consequently, the two curves cross, and above the 3 mg. level liver extract is much more stimulatory than casein. Acid production was used as the criterion for growth because the color of the larger amounts of liver extract added interferes with turbidimetric readings. The liver extract may contain a single stimulatory substance which is different from strepogenin or possibly a number of stimulatory substances, one of which may be strepogenin. The greater effect of liver extract as compared to casein does not appear to be due t glutamine, inositol, choline, nicotinamide, thymine, lecithin, oleic acid cozymase, cocarboxylase, malic acid, succinic acid, fumaric acid, or exces B vitamins (10 times the quantities given in Table I), since addition of 1 mg. each of those substances to tubes conta ning 2.5 mg. of trypsinize

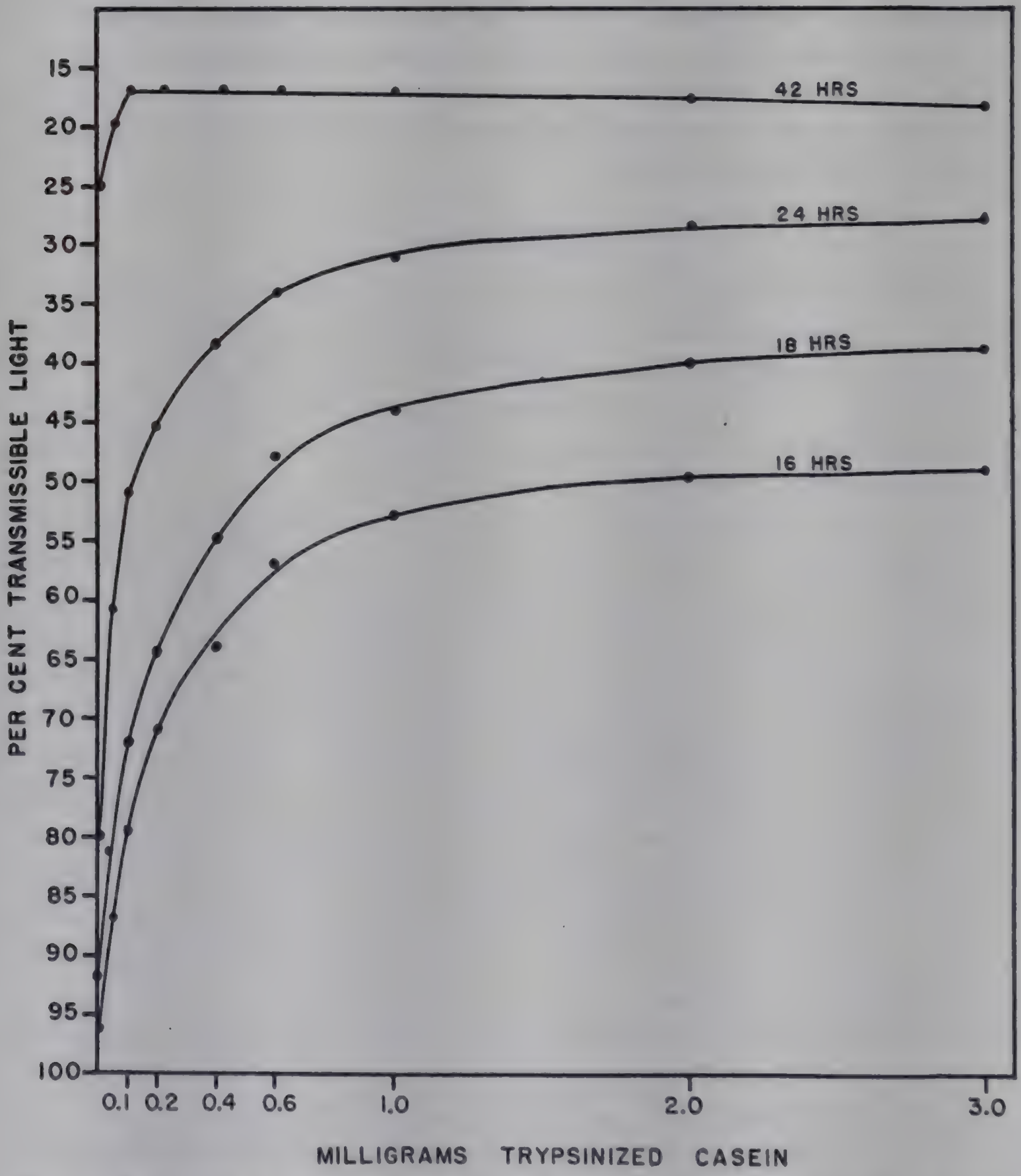

FIG. 1. Response of *Lactobacillus casei* to strepogenin as a function of time

casein did not appreciably increase growth above that obtained with the casein alone. Other trypsinized natural materials such as yeast extract, tankage, peptone, and distillers' solubles as well as the trypsinized purified proteins, soy bean glycinin, trypsinogen, tobacco mosaic virus, and insulin, when used in amounts of 5 to 50 mg. per tube, failed to give as much growth as liver extract.

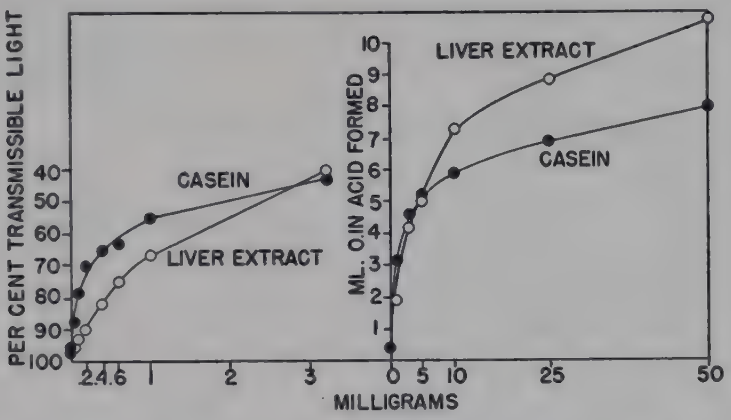

FIG. 2. Growth of *Lactobacillus casei* with liver extract and casein

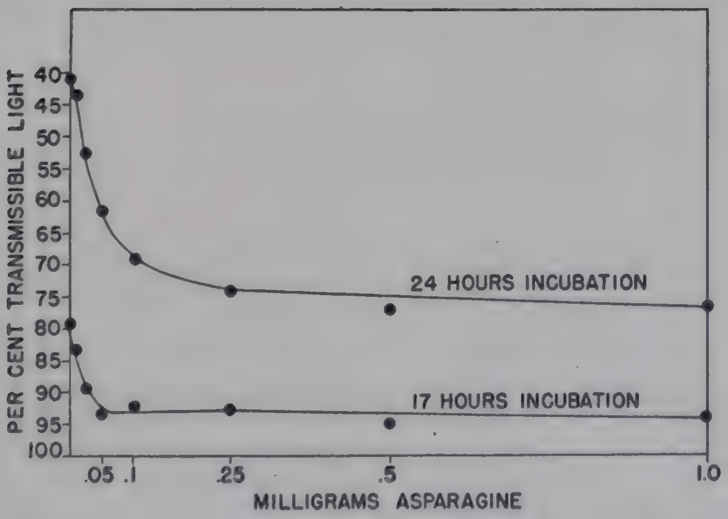

FIG. 3. Inhibition of growth of *Lactobacillus casei* by asparagine

Inhibition by Asparagine—Asparagine consistently inhibited growth. Iowever, the addition of as little as 0.05 mg. of trypsinized casein is sufficient to overcome the asparagine inhibition. The inhibitory effect of asparagine is illustrated in Fig. 3. At 17 hours, maximum inhibition occurred with 0.05 mg. of asparagine per tube, whereas at 24 hours 0.25 mg. of asparagine was required for maximum inhibition. Substitution of amino acids for the hydrolyzed casein in the basal medium (9) did not alter the inhibition by asparagine. In the completely synthetic medium the strepogenin curves were steeper and the blank tubes had consistently less growth than in the hydrolyzed casein medium. Glutamine, when substituted for asparagine, was not inhibitory, and tended to neutralize the inhibitory effect of asparagine.

Adaptations of Lactobacillus casei—An attempt was made to "train" *Lactobacillus casei* to accelerate its rate of synthesis of strepogenin and thus eliminate its requirement for added strepogenin. Serial subcultures were made daily by loop transfer in the basal medium (Table I) in the absence of added strepogenin. Growth was allowed to proceed for 24 hours prior to subculture in order to permit appreciable synthesis of strepogenin to occur, and turbidity readings were made after 16 hours to determine the degree of acceleration of strepogenin synthesis. Tubes inoculated with the usual peptone broth inoculum were included in this experiment as controls. After only five serial transfers, good growth was obtained in the strepogenin-free basal medium within 16 hours. Fig. 4 shows that the accelerated

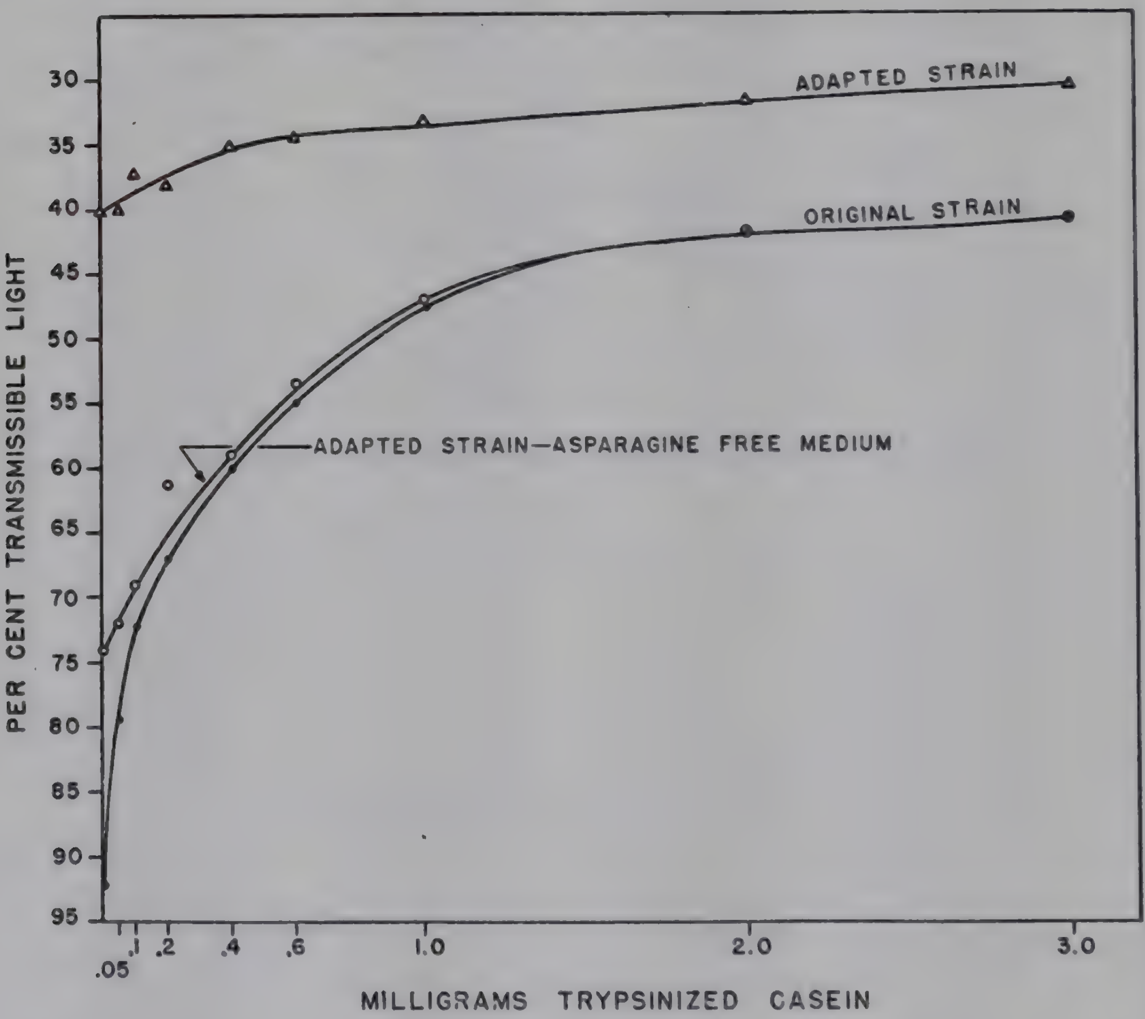

Fig. 4. Growth characteristics of *Lactobacillus casei* serially subcultured in the absence of strepogenin.

culture without added strepogenin grows even more rapidly than the original untrained culture supplied with 3 mg. of trypsinized casein. Interestingly, if asparagine is omitted from the basal medium, the accelerated strain again requires trypsinized casein for rapid growth to the same degree as the original culture. Approximately 0.4 mg. of asparagine per 10 ml. of medium is necessary for abundant growth of the adapted strain. Glutamine can replace asparagine but approximately 100 times more of glutamine than asparagine must be used. These findings again indicate a close metabolic relationship between asparagine and strepogenin.

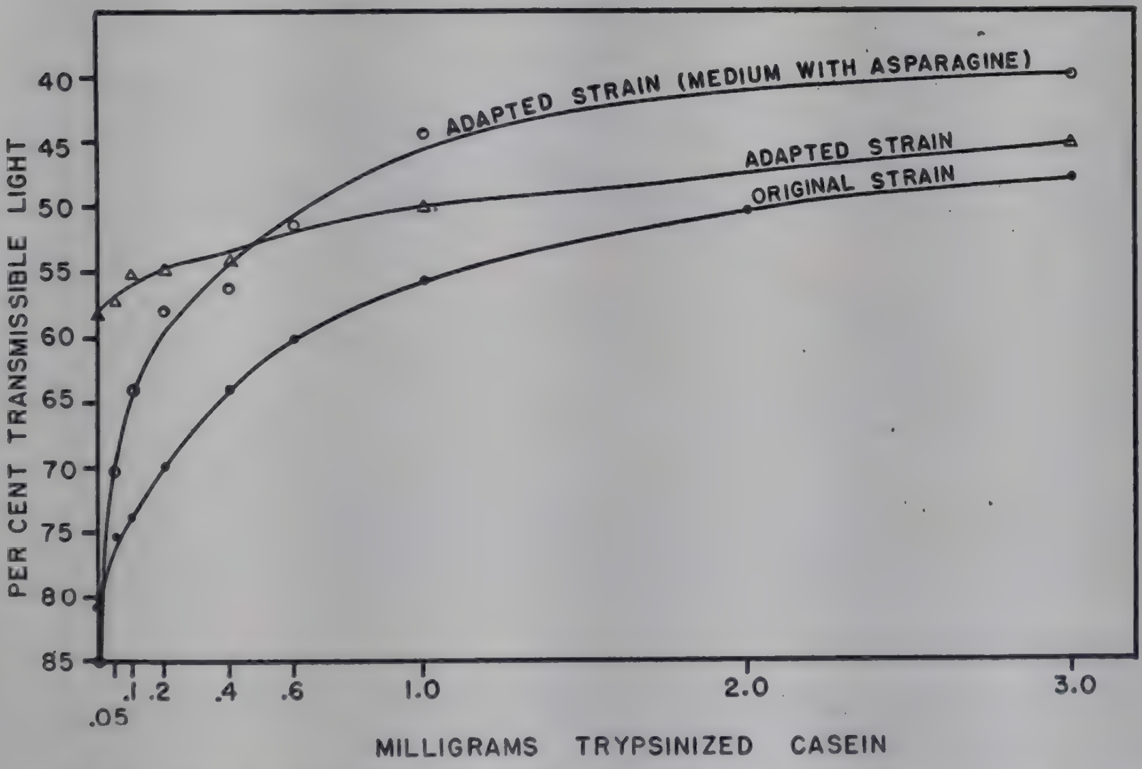

Fig. 5. Growth characteristics of *Lactobacillus casei* serially subcultured in the absence of asparagine and strepogenin.

By using the same technique as described above, except that a medium without strepogenin or asparagine was employed, it was possible to "train" *Lactobacillus casei* to grow fairly well within 16 hours without added asparagine or strepogenin (Fig. 5). However, in sharp contrast to the results with the previously mentioned adapted strain, if asparagine is added to the basal medium, the second adapted strain again requires trypsinized casein for rapid growth, as does the original culture. Thus, the inhibitory effect of asparagine for the original strain has been enhanced.

Inactivation and Reactivation of Strepogenin—Trypsinized casein which has been refluxed with hydrochloric acid at pH 1 (glass electrode) for 24 hours is completely inactive in asparagine-free medium, but is fully active when asparagine is supplied (Fig. 6). Quantitatively, 0.75 mg. to 1.0 mg. of L-asparagine per 10 ml. of medium is required for full restoration of the activity of 3 mg. of acid-inactivated strepogenin. Glutamine is also effective in this respect but approximately 100 times more of glutamine than asparagine is required. Biotin, glutathione, pyridoxal, β-alanine, and aspartic acid are ineffective in restoring activity. Asparagine also restores the activity of acid-refluxed strepogenin in the synthetic amino acid medium (9). Results similar to those with casein were obtained with acid-refluxed solubilized liver extract (Wilson's fraction L).

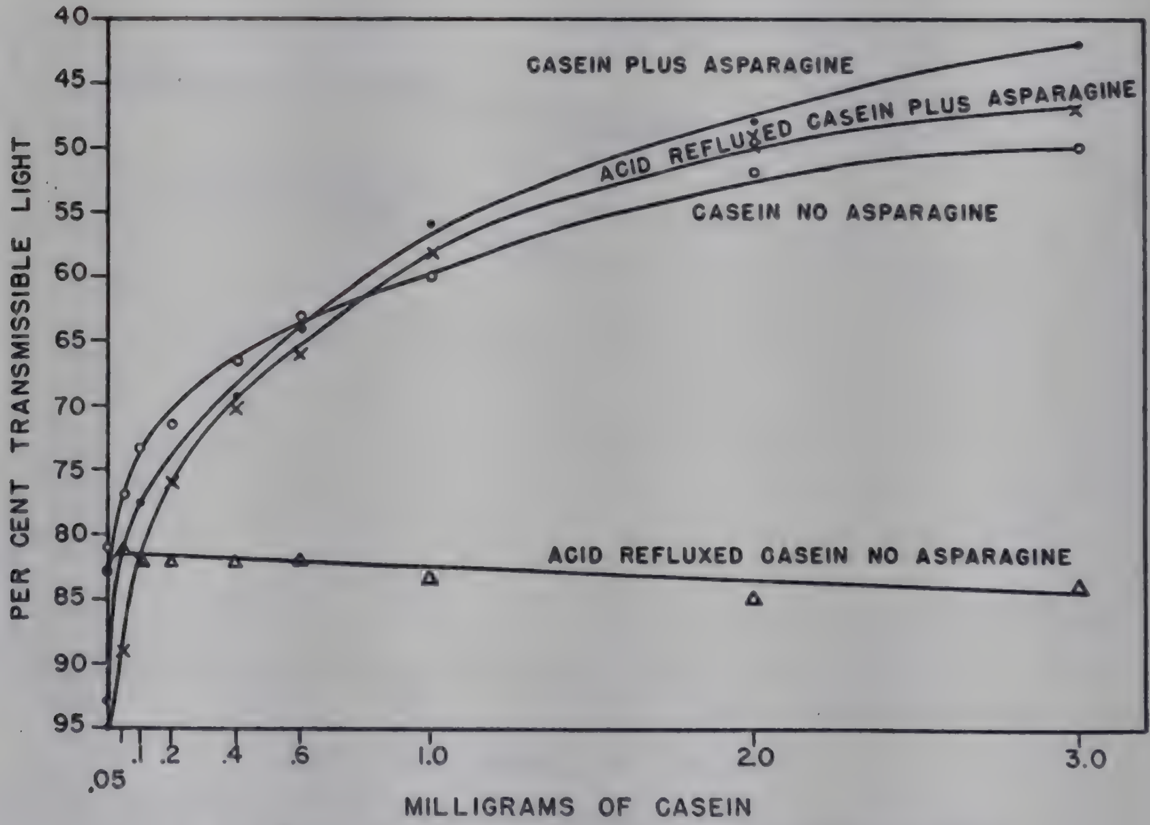

FIG. 6. Reactivation of acid-refluxed casein with asparagine

Trypsinized casein, which is kept at pH 1 at room temperature for 20 hours, shows no loss of activity. Refluxing of trypsinized casein at pH 10 results in complete loss of strepogenin activity, and, in contrast to the results at pH 1, the activity cannot be restored by asparagine. Refluxing of casein and liver extract at pH 7 results in partial loss of activity, which is restored by asparagine.

The adapted *Lactobacillus casei* culture, which can grow readily without strepogenin but requires asparagine, will not grow when the acid-refluxed casein is substituted for asparagine. This fact plus the fact that the normal *L. casei* strain requires asparagine for rapid growth with acid-treated casein would appear to indicate that the acid inactivation of trypsinized casein is due to hydrolysis of the amide group of asparagine, or of other amides which may perform a similar function.

These data emphasize further the close relationship between asparagine and strepogenin. The elucidation of its nature must await the isolation of strepogenin in pure form or at least free of asparagine.

SUMMARY

The effects of size, age, and growth medium on the response of the *Lactobacillus casei* inoculum to strepogenin are described. Trypsinized casein is about 4 times as potent as Wilson's liver fraction L in stimulating growth of *L. casei* when tested within the range of 0 to 1 mg. of each substance per 10 ml. of medium. However, at higher levels the liver fraction consistently supports more rapid growth than casein. Other trypsinized materials did not support as much growth as liver extract. Under the conditions employed, the growth of *L. casei* is markedly inhibited by asparagine, whereas glutamine is not inhibitory.

It was possible by serial subculture in strepogenin-free medium to adapt the strepogenin-requiring strain of *Lactobacillus casei* to grow rapidly without strepogenin. However, it is necessary to add asparagine to the medium for rapid growth of this adapted strain. It was also possible to adapt *L. casei* to grow rapidly without either strepogenin or asparagine. For this second adapted strain asparagine is inhibitory, and the inhibitory effect can be overcome by the addition of trypsinized casein to the medium.

Trypsinized casein which has been refluxed at pH 1 for 24 hours is completely inactive in asparagine-free medium, but is fully active when asparagine is added. Similar results were obtained with acid-refluxed Wilson's liver fraction L. Glutamine, in 100 times the quantity of asparagine, will also restore the strepogenin activity of acid-refluxed casein.

The data presented indicate strongly a close metabolic relationship between strepogenin and asparagine.

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ADDITIONAL OBSERVATIONS ON THE RATE OF GROWTH OF LACTOBACILLUS CASEI

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In the preceding paper (1) it was shown that the growth rate of *Lactobacillus casei* may be markedly affected by variations in the amount of asparagine present in the medium. This compound may be either stimulatory or inhibitory, depending upon the conditions existing; evidence was presented which indicates that asparagine is closely related metabolically to strepogenin. For example, strepogenin, in the form of trypsinized casein (2), is completely inactivated by mild acid hydrolysis when the test medium does not contain asparagine, whereas its activity is not affected if asparagine is supplied to the organism. Moreover, a culture of *Lactobacillus casei* "trained" by appropriate subculture to grow rapidly without strepogenin was found to require asparagine for rapid development; another "trained" culture grew well in the absence of both, but was markedly inhibited by asparagine. This effect was overcome by trypsinized casein.

As a result of these findings, experiments were undertaken to define more closely the nature of the relationship between asparagine and strepogenin, and to determine whether the rate of growth of *Lactobacillus casei* can be effectively altered by varying the level in the medium of known nutrients other than asparagine.

Experiments along those lines have led to the development of a chemically completely defined medium which is capable of supporting rapid growth of *Lactobacillus casei;* maximum turbidity is obtained in about 24 hours and maximum acid production in about 30 hours. The rate of growth of the organism, under these conditions, is not appreciably affected by trypsinized casein. Upon omission of the appropriate substance, the medium is suitable for the rapid turbidimetric or titrimetric assay of folic acid, biotin, and serine, and should be adaptable for the assay of other compounds required by the organism.

EXPERIMENTAL

In the first experiments conducted, the casein hydrolysate basal medium and inoculum described previously (1) were employed. Since asparagine decreases the rate of growth of *Lactobacillus casei* when added to this medium in the absence of strepogenin, it seemed possible that its inhibitory effect was due to an antagonism between the amide and some constituent of the basal medium. To test this hypothesis, an excess of each of the amino acids known to exist in casein and each of the vitamins and other supplements employed was added individually to the medium containing asparagine. Serine alone was found to be effective as a growth stimulant under these conditions. Approximately 2.0 mg. of serine, in addition to that present in the casein hydrolysate, are capable of overcoming the inhibition caused by 2.5 mg. of asparagine. However, larger amounts of serine stimulate more rapid growth than is obtained on the original asparagine-free medium. On the other hand, an increase in the serine level in the absence of asparagine is without effect. These results are summarized in Table I.

TABLE I

Effect of Asparagine and Serine* on Rate of Growth of Lactobacillus casei*

Supplement added	Turbidity, 17 hrs.
mg. per tube	*per cent light transmitted*
None†	76
10 mg. serine	76
2.5 mg. asparagine	93, 93
2.5 " " + 2 mg. serine	78, 79
2.5 " " + 10 " "	57, 56

* Throughout the work reported in this paper, L-asparagine, L-glutamic acid, and DL-serine were employed.

† Casein hydrolysate basal medium (1) without asparagine.

In the light of these findings, it appeared advisable to turn our attention to a medium in which the concentration of each constituent could be controlled. An amino acid basal medium patterned after that of Stokes, Gunness, Dwyer, and Caswell (3) was therefore employed in all of the remaining work. As before, the final volume was 10 ml. per tube. Except where otherwise indicated, the tubes containing the basal medium and the various supplements were sterilized by autoclaving at 115° for 12 minutes. After sterilization, the tubes were inoculated and incubated at 37° for the various times specified prior to determining their turbidities or titrating the acid produced. Throughout the work, a light inoculum similar to that described previously (1) was employed, except that 24 hour broth subcultures made daily from agar stabs were used to avoid any possible "training" effect of a week's serial subculture in the broth.

Effect of Amino Acids on Rate of Growth of Lactobacillus casei—The mutually stimulatory effects of serine and asparagine, when added to the basal Medium AA (Table II), were similar to those observed with the casein hydrolysate medium (1). Glutamine was not an effective substitute for asparagine. Moreover, it was found that the rate of growth in the presence of adequate amounts of serine and asparagine can be further increased by raising the glutamic acid level. Glutamine is capable of replacing glutamic acid when the amide is added at a level of about 0.5 to 1.0 mg. per tube; progressively larger amounts produce decreasing growth responses. These results are illustrated in Table III.

TABLE II

Composition of Amino Acid Basal Media

Constituent	Concentration	
	Media AA, B	Media B+, BA
	mg. per tube	*mg. per tube*
Amino acid mixture	*	*
L-Asparagine	†	2.5
DL-Serine	†	20.0
L-Glutamic acid	†	20.0
Dextrose (anhydrous)	100.0	100.0
Sodium acetate (anhydrous)	60.0	60.0
Adenine, guanine, uracil, each	0.1	0.1
Salts A		
K_2HPO_4	5.0	10.0
KH_2PO_4	5.0	10.0
Salts B		
$MgSO_4 \cdot 7H_2O$	2.0	4.0
NaCl	0.1	0.2
$FeSO_4 \cdot 7H_2O$	0.1	0.2
$MnSO_4 \cdot 4H_2O$	0.1	0.2
$MnCl_2 \cdot 4H_2O$	0.05	0.25
Ascorbic acid		2.5‡
	γ per tube	*γ per tube*
Riboflavin, thiamine HCl, nicotinic acid, pantothenic acid, each	2.0	4.0
Pteroylglutamic acid	0.02	0.1
Biotin	0.002	0.025
Pyridoxamine	4.0	10.0
Pyridoxine		10.0
Pyridoxal		10.0‡
p-Aminobenzoic acid	0.4	2.0
Inositol		100.0

* Amino acid basal mixture: Per tube 4 mg. of DL-tryptophan, 1 mg. of L-lysine, 2 mg. each of DL-alanine, L-arginine, DL-aspartic acid, L-cystine, glycine, L-histidine, L-hydroxyproline, DL-isoleucine, DL-leucine, DL-methionine, DL-norleucine, DL-phenylalanine, L-proline, DL-threonine, L-tyrosine, and DL-valine.

† The concentrations of L-asparagine, DL-serine, and L-glutamic acid were respectively in Medium AA, 2.5, 2.0, and 2.0 mg., and in Medium B, 2.5, 20.0, and 20.0.

‡ Pyridoxal was omitted from Medium BA and ascorbic acid from Medium B+.

When added to the basal medium supplemented with 20 mg. each of serine, asparagine, and glutamic acid per tube, none of the other amino acids in amounts up to 20 mg. per tube effected an appreciable increase in growth rate.

Effect of B Vitamins—Although *Lactobacillus casei* grows fairly rapidly on Medium B, which contains 2.5 mg. of asparagine and 20 mg. each of serine and glutamic acid, it was found possible to increase the growth rate appreciably, but erratically, by the addition of crude materials such as yeast extract, corn steep liquor, and liver. Trypsinized casein was less effective. Since in some experiments the effect of these crude supplements could be partially replaced by increased amounts of biotin plus vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine together, added aseptically), the levels of all of the vitamins and minerals were increased to the values shown under Medium B+ (Table II) to insure that sufficient quantities

TABLE III

Effect of Serine, Asparagine, and Glutamic Acid on Rate of Growth of Lactobacillus casei

Experiment No.	Amino acids added* per tube	Turbidity, 17 hrs.
		per cent light transmitted
910	20 mg. asparagine, 20 mg. glutamic acid, plus	
	2 mg. serine	94, 95
	10 mg. serine	75, 79
	20 " "	69, 70
	20 mg. serine, 20 mg. glutamic acid, plus	
	0 mg. asparagine	91, 91
	0.5 mg. asparagine	71, 70
	5.0 " "	68, 69
	20.0 mg. asparagine	62, 61
	20 mg. serine, 2.5 mg. asparagine, plus	
	2 mg. glutamic acid	81, 81
	10 mg. glutamic acid	72, 73
	20 " " "	70, 70
916	10 mg. serine, 1 mg. asparagine, 2 mg. glutamic acid, plus	
	No supplement	81, 81
	1 mg. glutamic acid	75, 76
	5 " " "	70, 72
	10 mg. glutamic acid	67, 68
	0.5 mg. glutamine	67, 68
	1 mg. glutamine	71, 71
	5 " "	82, 84
	10 mg. glutamine	88, 89

* The basal medium was Medium AA, minus serine, glutamic acid, and asparagine.

for optimum growth were present. Inositol was also included in the modified medium.

The relative growth-stimulating effects of corn steep liquor solids, yeast extract (Difco), and trypsinized casein, when added to the basal medium containing all of these supplements (Medium B+), are shown in Fig. 1.

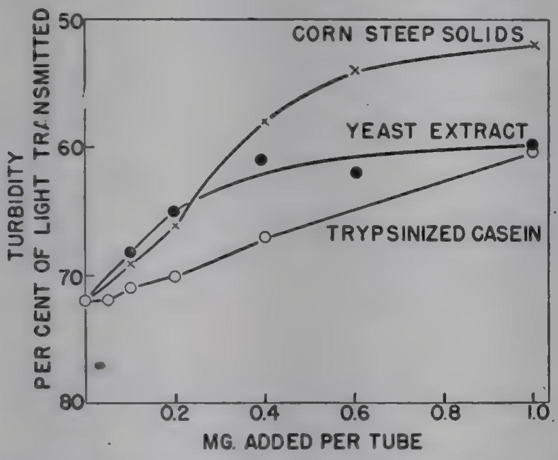

FIG. 1. Effect of crude supplements on the growth of *Lactobacillus casei;* basal Medium B+, 17 hours incubation.

TABLE IV

Effect of Added Reducing Agents on Growth Rate of Lactobacillus casei (Basal Medium B+)

Experiment No.	Supplement added per tube	Supplement sterilized by filtration and added aseptically	Supplement sterilized by autoclaving with medium
		per cent light transmitted	*per cent light transmitted*
1112	None	79	79
	0.5 mg. ascorbic acid	65	65
	1.0 " " "	56	56
	2.5 " " "	45	45
	1.0 " thioglycolic acid	66	60
	2.5 " " "	54	56
	2.5 " cysteine	70	70
23	None		59, 58
	2.5 mg. ascorbic acid		39, 40
	2.5 " *d*-isoascorbic acid		36, 36
	3.0 " corn steep solids		34
	3.0 " trypsinized casein		41

Turbidities were measured after 17 hours incubation. The relatively slight activity of the trypsinized casein suggests that the stimulation produced by corn steep solids and yeast extract is due largely to some factor other than strepogenin. Essentially the same results were obtained when pyridoxal was omitted, and the tubes containing basal medium and all of the supplements sterilized by autoclaving. This procedure was therefore followed in the remaining experiments, except those in Table IV.

Effect of Other Known Compounds—Since the growth rate of *Lactobacillus casei* on basal Medium B+ can be increased by the above-mentioned crude materials, it appeared advisable to test the possible effect of an additional series of known compounds. It was found that increasing by 5-fold the mineral level of the medium had no effect; xanthine was inhibitory at levels above 10 γ; hypoxanthine, guanosine, cytidylic acid, and yeast nucleic acid at levels up to 50 γ per tube, nicotinamide (0.5 to 3.0 γ per tube), and sulfuric acid-hydrolyzed casein (12.5 to 125 mg. per tube) were without effect. However, a mixture of 5 mg. each of fumaric, succinic, and ascorbic acids and sodium ethyl oxalacetate produced a definite response in growth rate. This was found to be due to the ascorbic acid content of the mixture. As might be anticipated, the effect of this compound appears to be due to its reducing properties rather than to a possible specific vitamin effect. The growth-accelerating effect of ascorbic acid is compared with that of other reducing agents in Table IV. It is apparent that cysteine is not effective at the levels used, while thioglycolic acid is less effective than ascorbic acid, and *d*-isoascorbic acid is equally potent.

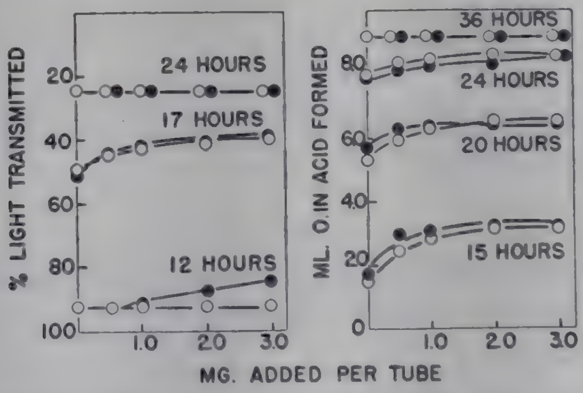

FIG. 2. Effect of trypsinized casein and corn steep liquor upon the rate of growth of *Lactobacillus casei*. Basal Medium BA; ○, trypsinized casein; ●, corn steep liquor solids.

Effect of Crude Materials on Rate of Growth of Lactobacillus casei on Improved Synthetic Medium BA—Since relatively rapid growth of *Lactobacillus casei* is attained on basal Medium BA (containing the ascorbic acid supplement), a careful check was made on the possible effect on the growth rate of the organism when trypsinized casein, a known source of strepogenin, is added. Growth response curves, both turbidimetric and titrimetric, for *Lactobacillus casei* with graded amounts of trypsinized casein and corn steep liquor were determined at varying incubation times. The tubes containing basal medium, ascorbic acid, and supplements were sterilized by autoclaving. Turbidities were determined after 8, 12, 14, 16, 17, 20, and 24 hours incubation and acid production in replicate sets of tubes after 15, 20, 24, 36, and 45 hours. The results of these experiments are summarized in Fig. 2. The curves for some of the time intervals are omitted for the sake of clarity. In addition, the rates of growth (turbidity) and of acid production by the organism in the basal Medium BA without crude supplements

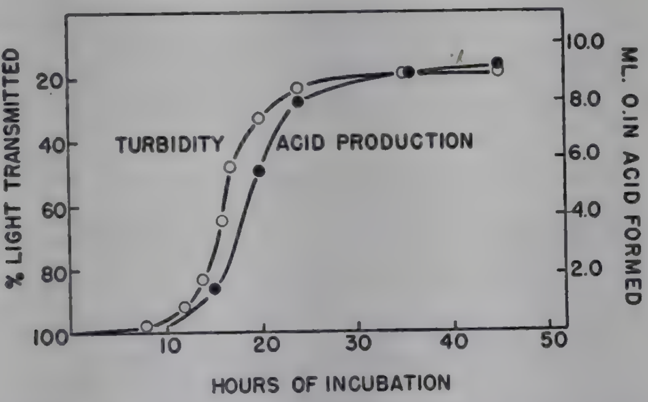

Fig. 3. Rate of growth of *Lactobacillus casei* on basal Medium BA; no supplements added.

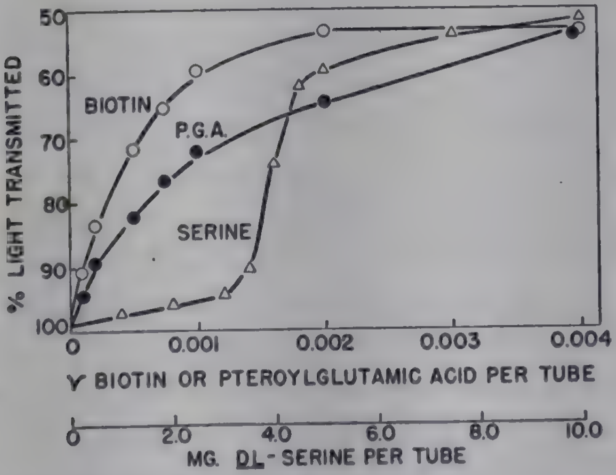

Fig. 4. Growth response of *Lactobacillus casei* to biotin, pteroylglutamic acid, and DL-serine. Basal Medium BA minus the compound tested; 17 hour incubation period.

are plotted in Fig. 3. It is of interest that there is only about a 3 hour lag between turbidity and acid production.

Use of Improved Lactobacillus casei Medium (BA) As Basal Medium for Amino Acid and Vitamin Assays—Medium BA gives constant and reproducible rapid growth which is not appreciably affected by the addition of crude materials likely to contain "non-specific" stimulatory substances. It should, therefore, be well suited, upon the omission of the appropriate nutrient, for the rapid and accurate assay of those vitamins and amino acids required by *Lactobacillus casei.* A few tests along these lines were carried out. Growth response curves (17 hour turbidity) for DL-serine, pteroylglutamic acid, and biotin are illustrated in Fig. 4. Similar curves were obtained by titrating the acid formed after 22 hours. While we have not had the opportunity to carry out assays of various source materials for comparison with literature values, it is felt that the present method should prove satisfactory, since the standard curves shown in Fig. 4 are highly reproducible. It appears probable that a more practical basal medium for assays of some of the vitamins could be developed with hydrolyzed casein instead of purified amino acids as the nitrogen source.

DISCUSSION

The chief factor limiting the *rate* of growth of *Lactobacillus casei* in a casein hydrolysate or amino acid medium of the usual type is presumably the rate of synthesis of strepogenin. It follows, therefore, that the increase in growth rate which is observed when additional quantities of serine, asparagine, and glutamic acid are supplied to the organism is due to an increase in the rate of strepogenin synthesis. These amino acids may function directly as precursors of strepogenin; glutamic acid and possibly serine have been postulated as components of the strepogenin molecule (4). Since DL-serine was employed in the present work, the possible rôle of D-serine remains to be investigated. Evidence presented previously (1) indicates that there is a close metabolic relationship between asparagine and strepogenin; asparagine may represent a component of the strepogenin molecule or alternatively may serve as an amide donor. The latter interpretation is consistent with the observation that mild acid hydrolysis destroys strepogenin activity in the absence of added asparagine, but is without effect when the amide is supplied to the organism (1).

The effectiveness of *l*-ascorbic acid and *d*-isoascorbic acid in further increasing the rate of growth of *Lactobacillus casei* is presumably due to their reducing effect. It appears unlikely that an appreciable fraction of the growth stimulation produced by the addition of trypsinized casein to the original medium can be attributed to a similar effect, since the amounts added are small.

The 17 hour growth response curve for DL-serine (Fig. 4) is of interest, in that the stimulatory effect of this amino acid under these conditions is not observed until amounts substantially larger than the requirement for full growth upon prolonged incubation are added. Thus, 2 mg. per tube are adequate if growth is allowed to proceed for 72 hours, whereas about 7 mg. per tube are required for an optimum *rate* of growth. It is possible that serine plays two distinct rôles in the nutrition of *Lactobacillus casei:* its direct utilization as a cell protein component and also as a possible precursor of strepogenin.

Some difficulty has been reported in the use of *Lactobacillus casei* for microbiological assays because of the effect of non-specific stimulatory substances on the growth of the organism (5). It is believed that improvement of basal assay media along the lines indicated in the present paper would resolve these difficulties to a considerable extent.

SUMMARY

A chemically defined complete medium has been developed which permits the rapid growth of *Lactobacillus casei*. Essentially maximum turbidity is obtained at about 24 hours and maximum acid production at about 30 hours.

The organism does not grow at an appreciably more rapid rate when trypsinized casein, a known source of strepogenin, is added to the improved medium.

The pronounced increase observed in the growth rate of the organism when the levels of asparagine, serine, and glutamic acid are increased is consistent with the view that these amino acids may serve directly or indirectly as precursors of strepogenin. Serine is stimulatory in an amount substantially greater than the requirement for growth upon prolonged incubation.

The improved medium, upon omission of the appropriate nutrient, is capable of serving as a basal medium for the rapid turbidimetric or titrimetric assay of substances required for the growth of *Lactobacillus casei*.

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APPLICATION OF PAPER CHROMATOGRAPHY TO THE ESTIMATION OF SOME FREE AMINO ACIDS IN TISSUES OF THE RAT*

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The development of paper chromatography by Consden, Gordon, and Martin (1) has opened a new approach to the problem of resolving and detecting small amounts of substances in a complex biological mixture. Although this technique was originally designed for the qualitative analysis of protein hydrolysates, a number of publications have appeared in which numerous adaptations and improvements are described (2) as well as attempts to use paper chromatography quantitatively (3–5).

The present work was undertaken to ascertain the applicability of this procedure to the study of amino acid metabolism.

Methods

Tissue extracts were prepared free of proteins and lipides, as recently described (6). Chromatographic analyses of extracts were carried out in accordance with the modification of Williams and Kirby (7). For quantitative estimation of amino acids, 0.1 ml. of extract was used, divided into five approximately equal small spots applied at 2 cm. intervals on Whatman filter paper No. 4. Chromatography was carried out for 18 hours with redistilled phenol saturated with water. Amino acids were located on the chromatogram with a 0.05 per cent ninhydrin solution in butanol. The spots developed on the chromatogram were cut out of the paper, placed in test-tubes, and 2 ml. of a 1 per cent ninhydrin solution added. The addition of 1 ml. of a 10 per cent aqueous pyridine solution was found favorable. The tubes were placed on a water bath for 20 minutes. Full color development was obtained at the end of this period. The colored solution was transferred to a 25 ml. volumetric flask, made to volume, and read in the Beckman spectrophotometer at 570 mμ. Spectrophotometric readings were compared with readings obtained for known solutions of amino acids treated in a similar manner.

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EXPERIMENTAL

Fig. 1 indicates that the four fractions found in rat liver fall within the range of aspartic acid, glutamic acid, glycine, and alanine. Other amino acids were present in lower concentrations and a quantitative estimate was impossible. It can be observed that aspartic acid and glutamic acid are free of interference from any of the amino acids under study. Glycine is shown to be well separated from threonine and to overlap slightly taurine and serine. Below the alanine spots are shown ten amino acids which do not interfere with any of the four fractions found in rat liver extracts.

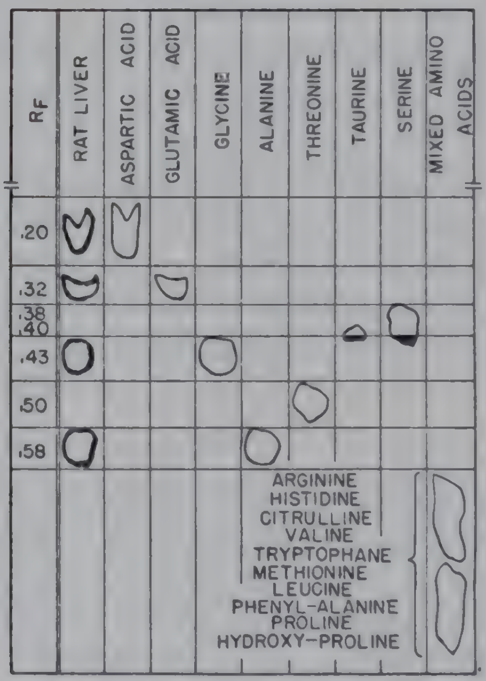
RF
RAT LIVER
ASPARTIC ACID
GLUTAMIC ACID
GLYCINE
ALANINE
THREONINE
TAURINE
SERINE
MIXED AMINO ACIDS
.20
.32
.38
.40
.43
.50
.58
ARGININE
HISTIDINE
CITRULLINE
VALINE
TRYPTOPHANE
METHIONINE
LEUCINE
PHENYL-ALANINE
PROLINE
HYDROXY-PROLINE

Fig. 1. Paper chromatogram of known amino acid solutions and rat liver extract

Possible interference from peptides, glutamine, and asparagine was taken into consideration. The presence of peptides in any of the four fractions was ruled out by elution of the individual fractions followed by acid hydrolysis (8). No changes were observed upon chromatography of the hydrolysates. Asparagine was shown to have the same R_F value as glycine. The presence of asparagine was ruled out by the failure to demonstrate aspartic acid upon hydrolysis of the glycine fraction. Glutamine, which moves at the same rate as alanine, in the chromatogram, is converted into pyrrolidonecarboxylic acid during the extraction procedure The latter compound does not interfere, as it does not appear in the chromatogram.

The reproducibility of the method was shown by analysis of an alanine solution containing 100 γ of amino nitrogen per ml. The results of twenty separate determinations showed an average of 103 γ of amino nitrogen, with a coefficient of variation of 5 per cent.

Paper chromatography, in its present state, offers numerous possibilities as a quantitative method in the study of amino acid metabolism. In this laboratory it has been used in studying the effect of adrenalectomy on the amino acid distribution in liver. It has also been found of great value in demonstrating the conversion of histidine to glutamic acid by the action of rat liver extracts. Table I shows the relative concentration of aspartic acid, glutamic acid, glycine, and alanine in normal rat liver. Extension of this procedure to kidney, spleen, heart, and skeletal muscle indicates that the same four amino acids exist in these tissues in similar concentrations.

TABLE I

Distribution of Amino Acid Nitrogen in Some Fractions of Liver

Animal No.	Amino acid nitrogen per gm. fresh tissue			
	Aspartic acid	Glutamic acid	Glycine	Alanine
	γ	γ	γ	γ
1	50	57	80	86
2	47	77	100	120
3	68	64	150	85
4	43	62	120	74
5	61	50	85	68
6	50	56	80	78
7	72	92	110	70
8	48	45	100	60
9	46	60	90	82
10	54	54	95	70
Average.....	53.9	61.7	101.0	79.3

SUMMARY

Paper partition chromatography has been applied to the estimation of some amino acids present in rat liver extracts. The presence of glutamic acid, aspartic acid, glycine, and alanine has been demonstrated. A quantitative estimation of these fractions was made possible by spectrophotometric analysis of the colored solution produced by ninhydrin.

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STUDIES ON THE PREPARATION OF SOY BEAN PROTEIN FREE FROM PHOSPHORUS

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(Received for publication, October 21, 1948)

It is well known that the properties and nitrogen content of isolated soy bean protein vary with the method of preparation (1–3). The present study was undertaken for the purpose of determining the phosphorus content of soy bean protein prepared by various known methods and to find a method of isolating phosphorus-free soy bean protein suitable for fundamental studies.

Averill and King (4) determined the phytin phosphorus content of several varieties of soy beans, and found a variation from 0.505 to 0.727 per cent. Earley and DeTurk (5) pointed out that the phytin content of corn increased during maturation. Earle and Milner (6) attributed approximately 80 per cent of the total phosphorus in the Dunfield variety of soy beans to phytin. Spitzer and Phillips (7) reported that phytin phosphorus in samples of solvent-extracted and screw-pressed soy bean meal (variety not given) accounted for only 58 per cent of the total phosphorus. Fontaine, Pons, and Irving (1) were the first to point out the importance of phytin as an impurity in seed protein preparations. It was shown that, when proteins were peptized from seed meals at a definite pH and later precipitated by adjusting the pH to the isoelectric region, a predictable amount of phytic acid phosphorus would be precipitated with the protein. Evidence was presented which suggested the formation of a protein-phytic acid complex, the dissociation of which depended somewhat on pH and solubility. It was also shown that the phosphorus content of isolated protein preparations was practically independent of their ash content.

Materials and Methods

Beans—Except for pilot plant studies, Illini soy beans, 1943 crop, were used throughout this study. The beans were cracked, dehulled by forced air separation, and flaked to a thickness of 0.005 to 0.007 inch in preparation for oil extraction. Prior to removal of the oil, the flakes contained 7.00 per cent nitrogen and 0.585 per cent phosphorus, 4.5 per cent of which was inorganic phosphorus. To insure against loss of significant phosphorus by dehulling the beans, the seed parts from a separate sample were separated by hand and analyzed for phosphorus. The percentage of total phosphorus attributed to each part of the bean was hulls 1.65, embryo 2.60, cotyledons 95.75. Oil was extracted with a Soxhlet extractor. During the course of the investigation, extractions were made with hexane, absolute and 95 per cent ethanol, diethyl ether, dioxane, methyl and ethyl formate, and an 80:20 benzene-alcohol mixture. In no case could the removal of phytic acid phosphorus be detected. Hexane-extracted dehulled flakes contained 9.08 per cent nitrogen and 0.742 per cent phosphorus.

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Total Phosphorus—The sample was digested with sulfuric, nitric, and perchloric acid essentially in accordance with the method of Gerritz (8). Phosphorus was then determined colorimetrically, as described by Truog and Meyer (9). Since the isolated proteins were free from inorganic phosphorus and contained only a small amount of phosphatide phosphorus, it was assumed that the phosphorus found was primarily of phytin origin.

Inorganic phosphorus was determined by the method of Earle and Milner (6).

Nitrogen was determined by the Kjeldahl-Gunning-Arnold method, by using a mercury catalyst and a 1 hour digestion after it had become clear. By digesting soy bean protein preparations for periods up to 7 hours, Smiley and Smith (2) have shown that a digestion period of 1 hour is sufficient to recover all of the nitrogen. All analytical determinations are reported on a moisture-free basis. Corrections for ash have been made only where indicated.

Preparation of Protein—With the exception of variations noted under "Experimental," the following procedure was employed to isolate protein from the oil-free meal. The protein was peptized from the flakes or leached flakes by suspension in 20 parts by weight of the desired solvent, the mixture being stirred for 1 hour with a motor-driven stirrer. The clear extract was then removed by centrifuging for 6 minutes at approximately 2000 × *g*. The residue was taken up with the same quantity o dispersing solution as used in the first extraction and the procedure repeated. A third extraction was carried out in the same manner. The three successive extractions were made to insure removal of as much protein as possible with the particular dispersing agent being used. The procedure for recovering the protein from the combined extracts depended upon the dispersing agent. In extractions with water or alkaline solutions the preparation was precipitated by adjusting the pH to the point o minimum solubility, followed by centrifuging. The curd was then washed free from excess salt by suspension in water and centrifuging. When salt were used as the dispersing agent, the extract was placed in a cellophane bag and dialyzed at 5° until free from salt. Unless otherwise stated, th protein preparation was recovered from the dialyzer contents by adjusting the pH to the point of minimum solubility of the protein. The procedures used for recovering protein from extracts of organic dispersing agents are described under "Experimental."

EXPERIMENTAL

Water or Alkali Peptization and Acid Precipitation—The most economical method of isolating soy bean protein from the oil-free meal is that of extracting with water or dilute sodium hydroxide, clarifying the extract by centrifugation, and precipitating by adjusting the pH to the region of minimum solubility with acid. The results listed in Table I are representative of preparations isolated by this procedure. The pilot plant run involved only a single extraction with a solvent to meal ratio of 20:1. Details of the pilot plant operation are reported elsewhere (10).

In general, the phosphorus contents of the isolated protein preparations were those predicted from the peptization curves of Fontaine *et al.* (1). For example, when extractions were made at pH 6.6, 87 per cent of the phosphorus would be expected to combine with 82 per cent of the protein, and when extractions were made at pH 9.5, 82 per cent of the phosphorus would be expected to combine with 92 per cent of the protein. Also, precipitations made at pH 5.0 would be expected to contain approximately 45 per cent of the total phosphorus.

During the pilot plant operations, it was noted that the solid cake which collected in the centrifuge bowl during clarification of the alkaline extracts contained from 1.47 to 2.48 per cent phosphorus (5 to 9 per cent phytic acid (P × 3.55). By employing Anderson's method (11), barium phytate was readily isolated from this material, substantiating an earlier report (1) that part of the cloudiness observed in soy bean protein extracts is attributable to phytin.

Leaching Soy Bean Meal with Acids at pH 4.2—The practice of leaching solvent-extracted soy bean flakes with aqueous solutions adjusted to the isoelectric region of the protein prior to extracting the protein has for its main purpose that of inhibiting the "browning reaction." For this reason, the acidity is preferably obtained with sulfur dioxide (12, 13). Other desirable properties for "protein" prepared in this manner have been claimed (14).

In his early work, Anderson (11) used 0.2 per cent hydrochloric acid solution to leach phytin from seed meal. However, he learned that phytase remained active in concentrations of less than 1 per cent acid and prevented the isolation of a crystalline phytate. Since the concentration of acid required to maintain a pH of 4.2 in the presence of soy bean meal is of the order of 0.2 per cent, it was desired to investigate the re-

TABLE I

Phosphorus and Nitrogen Content of Soy Bean Protein Extracted with Water or Alkali and Precipitated with Acid

Solvent for oil extraction	Peptizing agents*	pH of extract	Special treatment	Pptn.		N†	P†	Ash	Yield N
				Acid	pH				
						per cent	*per cent*	*per cent*	*per cent*
Hexane	H_2O	6.6	Curd washed, dissolved at pH 8, clarified, repptd., washed	H_2SO_4	4.2	15.52	1.06	0.30	70
"	"	6.6	Curd washed, redissolved, repptd.	"	4.2	15.58	1.00	1.60	65
"	0.1% NaOH	10.0	Pilot plant run‡	SO_2	4.2	15.45	0.76		44
"	0.1% "	9.5	Washed once	"	4.2		0.75		73
"	0.1% "	9.5	Solution adjusted to 0.8% NaOH, pH 11.5, stood 17 hrs. at 5°	"	4.2		0.64		
"	H_2O	6.6	Curd washed once	HCl	5.0	15.04	0.46		50
Absolute EtOH	"	6.6	" " "	"	5.0	16.40	0.31		15
" "	"	6.6	" " "	"	4.2		0.76		Low
95% EtOH	"	6.6	" " "	"	4.2		0.96		"

* Distilled water was used throughout, except for the pilot plant preparation.

† Nitrogen and phosphorus analysis corrected for moisture only.

‡ Batch extraction with 100 pounds of commercial solvent-extracted "brew" flakes of high water-soluble nitrogen.

moval of phosphorus by leaching at the pH of minimum solubility of the protein. The results are shown in Table II. Leaching removed nearly one-third of the total phosphorus. Protein prepared from the leached meal by extracting with sodium hydroxide at pH 10 contained 0.67 per cent phosphorus and 15.56 per cent nitrogen. The amount of phosphorus removed by leaching agrees closely with the peptization curves of Fontaine *et al.* (1), which show 27 per cent of the phosphorus and 9 per cent of the nitrogen to be soluble at pH 4.2.

Leaching the curd precipitated from an alkali extract with 0.5 N trichloroacetic acid, followed by adjusting to pH 4.2 and washing, yielded a preparation of protein containing only 0.33 per cent phosphorus; however, the nitrogen content was low (14.50 per cent), indicating degradation.

TABLE II

Removal of Total Phosphorus and Nitrogen by Leaching Solvent-Extracted Soy Bean Flakes with Sulfurous Acid Solution Adjusted to pH 4.2

Leaching	Solids extracted	Nitrogen extracted	Phosphorus extracted
	per cent	*per cent*	*per cent*
1st leach, 1 hr.	18.7	4.4	17.2
2nd " 24 hrs.	6.2	1.6	6.2
3rd " 24 "	4.2	2.1	3.4
4th " 24 "	4.1	1.4	2.1
Total	33.2	9.5	28.9

Precipitation of Calcium or Barium Phytate from Alkaline Protein Dispersions—The work of Fontaine *et al.* (1) indicated that the protein-phytin complex is dissociated in alkaline dispersions. If this dissociation occurs to any extent, it should be possible to precipitate the insoluble calcium or barium phytate from the dispersions. In this connection, it is well known that a precipitate occurs when lime is added to an alkaline meal extract (15, 16). Osborne and Campbell (17) reported that best results were obtained when barium hydroxide was used with sodium chloride to extract the protein from soy bean meal.

A series of experiments was performed for the purpose of determining whether or not phytin could be precipitated from an alkaline protein extract by the addition of calcium or barium ions. Approximately 80 per cent of the meal nitrogen was recovered in the isolated protein. 3 per cent of the total phosphorus remained in the insoluble residue on extraction with 0.2 per cent sodium hydroxide and 8 per cent on extraction with 0.1 per cent calcium hydroxide. The nitrogen values for the isolated preparations varied in accordance with the severity of the alkali treatment, probably as a result of the loss of amide nitrogen. The results of the experiments are tabulated in Table III. For convenience, Table III is divided into two parts. The first part shows the results of attempts to precipitate barium phytate from the alkaline extracts. The small barium precipitate obtained in each experiment was high in phosphorus and the isolated protein contained appreciably less phosphorus than protein obtained without the barium treatment (see Table I), indicating removal of phytate from the meal extract. However, the separation was not quantitative, even with an excess of barium ions present, indicating that not all of the phytate was free. The second part of Table III reveals that, when protein is extracted with either lime or barium hydroxide and precipitated in the normal manner, the protein may be expected to contain 0.8 to 1.00

TABLE III

Precipitation of Phytate from Soy Bean Meal Extract with Calcium and Barium Ions

Peptizing agent	pH of extract	Special treatment	P in Ca or Ba ppt.	P in protein
			per cent	*per cent*
0.2% NaOH	9.7	0.2% $Ba(OH)_2$ added, pH 10.9; centrifuged to remove ppt.	1.58	0.46
0.2% "	9.7	0.2% $Ba(OH)_2$ added; adjusted pH to 9.5 and heated to 80°; centrifuged	2.74	0.27
NaOH	10–11	Used meal after leaching with 0.5 N Cl_3CCOOH; 0.2% $Ba(OH)_2$ added to alkaline dispersion; centrifuged	2.55	0.22
0.1% NaOH	9.5	5% $BaCl_2$ added; stood 17 hrs. at 5°; centrifuged; Na_2SO_3 added to remove Ba; clarified and pptd.	1.90	0.18
0.1% "	9.5	5% $BaCl_2$ added; stood 17 hrs. at 5°; centrifuged; dialyzed	1.52	0.32
0.1% $Ca(OH)_2$	9.2	Pptd. at pH 4.2 with HCl		1.00
0.1% " *	9.6	Pilot plant run; pptd. with SO_2		0.79
0.2% $Ba(OH)_2$	8.4	Curd redissolved in NaOH and repptd.		1.00
1% $Ba(OH)_2$		Extract set 2 hrs., centrifuged	2.01	0.25
0.1% $Ca(OH)_2$	9.2	0.7% NaOH added; stood 17 hrs. at 5°, pH 11.5; centrifuged; dialyzed	5.60	0.20
0.1% "	9.2	Heated to 85°; centrifuged (ppt. discarded)		0.65

* Single extraction; all the others received three successive extractions.

per cent phosphorus. In other words, phytin does not remain with the insoluble residue upon filtering the meal extract. The fact that the addition of alkali to a lime extract produces a precipitate containing 5.60 per cent phosphorus indicates that the protein-phytin complex is dissociated to a greater extent at a higher pH, thus enabling the insoluble calcium phytate to be precipitated from the extract liquor.

Mild Hydrolysis with Sodium Hydroxide—For industrial use, soy bean protein is ordinarily given a mild hydrolytic treatment, usually by the action of dilute alkali on the meal extract (14–16, 18, 19). Such a treatment enhances the adhesive strength when the protein is dispersed in dilute alkali for use as a paper coating binder (20). This action of dilute alkali is not entirely understood, but, since ammonia and hydrogen sulfide are liberated during the treatment, it is probable that amide and disulfide groups are attacked.

A series of preparations were made to determine the phosphorus content after different degrees of mild alkali treatment. 100 gm. of hexane-extracted soy bean flakes were ground in a hammer mill and extracted successively with 1000 and 500 ml. of distilled water. The clarified solution (about 1300 ml.) was divided into 7 parts and sodium hydroxide added to the following respective concentrations: 0, 0.2, 0.6, 0.8, 1.0, 1.5, and 2.0 per cent. After each test solution had been digested for 17 hours at room temperature, the protein was precipitated at pH 4.2 with hydrochloric acid and the curd washed twice with water.

Nitrogen values for the series varied from 15.98 per cent for the unhydrolyzed to 14.60 per cent for the sample hydrolyzed with 2 per cent sodium hydroxide solution. The phosphorus contents for the series are shown in Fig. 1, indicating further that the protein-phytin complex is broken up to a greater extent by stronger alkali.

When protein containing 0.75 to 1.00 per cent phosphorus was dissolved in sodium hydroxide solution at pH 11.0 to 11.5, a slimy precipitate remained which was removed by centrifuging at 2000 × g. This precipitate invariably contained from 2 to 5 per cent phosphorus, 3 to 7 per cent nitrogen, and gave a strong Molisch test. The resulting protein (dry, ash-free basis) contained less phosphorus (0.3 to 0.5 per cent) and 14.6 to 15.2 per cent nitrogen.

Peptization with Organic Solvents—To determine whether or not organic solvents would peptize protein in preference to phytin, a number of extractions were made and the protein isolated. The recovery of the protein from organic solvents is not an entirely satisfactory process. From formic acid three procedures were followed: (*a*) dilution to 5 volumes and neutralization to pH 4.2 with cold 20 per cent sodium hydroxide, and redissolving and reprecipitating the curd twice, washing well each time to obtain protein free of salt (sodium formate); (*b*) dilution to 2 volumes and addition of 1 N trichloroacetic acid to precipitate all the protein, taking up the protein with water and adding sodium hydroxide to pH 4.2, and finally redissolving and reprecipitating the curd twice with thorough washing to remove the salt; (*c*) addition of 6 volumes of acetone to the formic acid extract to precipitate the protein, washing well with acetone to remove formic acid.

Protein was recovered from the formamide extract by dilution and adjusting the pH to 4.2. From acetic acid, the trichloroacetic acid method described above was used.

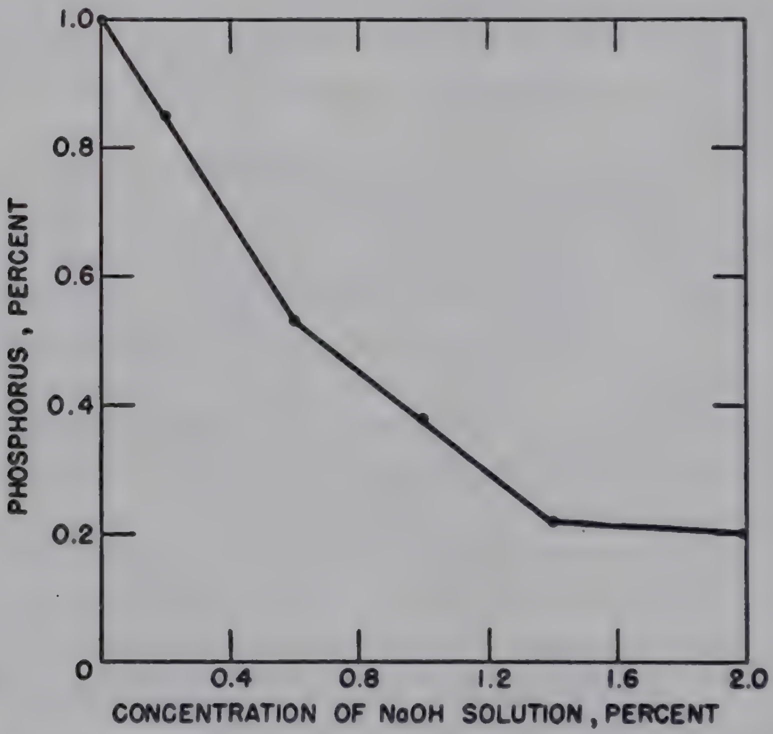

FIG. 1. Phosphorus contents of preparations of protein from meal extracts which had been digested for 17 hours in various concentrations of sodium hydroxide.

The phosphorus contents of protein preparations extracted from the meal with anhydrous organic solvents are reported in Table IV.

An average yield of 80 per cent of the nitrogen in the meal was obtained as protein (N = 15.16 per cent) with formic acid in the three experiment carried out. Both acetic acid and formamide gave approximately 20 per cent yields. Formic acid gave a dark brown extract and a white residue containing 0.30 per cent phosphorus. Attempts to decolorize the extract with activated carbon failed because efforts to remove the peptized carbon were unsuccessful.

Protein obtained from the formic acid extract by neutralization with sodium hydroxide repeatedly contained very little phosphorus. Analysi of the formic acid extract showed the presence of 90 per cent of the total phosphorus; therefore, if all of the phosphorus in the extract had reacted with the protein, the protein would have contained 0.95 per cent phosphorus. Since the major portion of the phosphorus combined with the protein upon precipitation with acetone, it becomes obvious that the presence of the large amount of salt produced upon neutralizing the formic acid was a factor in the recovery of protein substantially free of phosphorus.

Dissociation of Protein-Phytin Complex with Salts—Further evidence of the dissociation of the protein-phytin complex by salts was contained in the observation that, when soy bean protein, suspended in aqueous 2 M sodium acetate, was acetylated by passing ketene into the suspension, the acetylated protein contained only 0.04 per cent phosphorus When protein was allowed to react with formaldehyde in the absence of buffers, no reduction in phosphorus content was observed. When the reaction was carried out in the presence of 2 M sodium acetate, the phosphorus content was reduced from 1.0 to 0.21 per cent.

TABLE IV

Phosphorus Content of Protein Preparations Extracted from Soy Bean Meal with Anhydrous Organic Solvents

Peptizing agent	Pptn. method	Phosphorus in protein
		per cent
87% HCOOH	Dilute; neutralize to pH 4.2	0.04
87% "	" add 1.0 N Cl_3CCOOH to pH 1.0	0.16
87% "	Add 6 volumes acetone	0.82
CH_3COOH, glacial	Dilute; add 1.0 N Cl_3CCOOH to pH 1.0	0.52
$HCONH_2$	" adjust pH to 4.2	0.31

Osborne and Campbell (17) isolated the proteins from soy beans by using the classical method of extracting with sodium chloride solution and precipitating with saturated ammonium sulfate. These workers reported analyses for carbon, hydrogen, nitrogen, sulfur, oxygen, and ash, but did not mention phosphorus. Since their globulin fraction (glycinin) contained a high percentage of nitrogen, it is possible that phosphorus was absent; hence the reason for not reporting it. To check this point, several extractions were made by the procedure of Osborne and Campbell. Starting with the dehulled oil meal, analyses were made at each step to determine the percentage of total phosphorus removed in each operation. In general, the procedure consisted of extracting the dehulled oil flakes with hexane, extracting the protein from the oil-free flakes with 5 parts of 10 per cent sodium chloride solution, removing the insoluble residue, precipitating the protein by adding ammonium sulfate to saturation, filtering dialyzing against distilled water at 5°, redissolving the dialyzer contents redialyzing, and filtering the precipitated protein. The results are shown in Column A, Table V.

Column B, Table V, contains the results of an extraction carried out in the same manner as that of Column A, except that three successive extractions were made with a solvent to meal ratio of 20:1.

It is evident from the data in Table V that the greater portion of the phosphorus is left behind when the protein is precipitated with ammonium sulfate. Less than 5 per cent of the total phosphorus contained in the oil meal appeared in the glycinin. One-fifth of this phosphorus wa extracted with 80:20 benzene-alcohol, indicating the presence of phosphatides. The particular glycinin preparations reported in Table V sti contain a small amount of phosphorus. However, these may be considered to be crude when compared with Osborne's preparations. For example, where 41.2 and 39.9 per cent of the total nitrogen were obtaine as protein by us, he recovered approximately 21 per cent in one experimen and 12 per cent in another. He further purified his "crude" protein b dissolving it in brine, filtering, and redialyzing. The best product obtained by our use of this technique is described as follows: Yield (p cent N) 27.7; N = 16.80; P = 0.04 per cent (dry, ash-free basis).

TABLE V

Total Phosphorus and Nitrogen Removed by Various Steps in Preparation of Protein by Salt Extraction and Ammonium Sulfate Precipitation

Step in procedure	5:1 ratio* A		(20:1) × 3 ratio* B	
	per cent P	*per cent N*	*per cent P*	*per cent N*
Hexane extract	1.4	Trace	2.3	Trace
Meal residue after protein extraction	25.4	22.3	8.9	17.4
$(NH_4)_2SO_4$ filtrate after removal of protein curd	41.7		68.5	
Loss, 1st dialysis	11.9		7.7	
" 2nd "	0.7		5.7	
Glycinin or dialyzer ppt.	4.6	41.2	4.2	39.9
Total†	85.7	63.5	97.3	57.3

* Solvent to meal.

† Of the dialyzer content, only the precipitate (glycinin) was analyzed. Considerable nitrogen and some phosphorus remained in solution. The presence of ammonium sulfate precluded Kjeldahl analysis of each step; hence the large amount o nitrogen not accounted for.

To investigate further the dissociation of the protein-phytin complex by salts, a series of experiments was performed in which the protein curd, obtained by extracting with 0.1 per cent aqueous sodium hydroxide and precipitating with acid at pH 4.3, was leached with a saturated solution of sodium sulfate. The procedure was as follows: The wet protein curd was extracted three successive times by stirring for 1 hour with 10 parts of saturated solution of sodium sulfate, based on the weight of the solvent-extracted flakes; the pH during the sodium sulfate extraction was varied with either hydrochloric acid or sodium hydroxide, as required; the leached curd was finally dialyzed against distilled water at 5° to remove the salt.

TABLE VI

Removal of Total Phosphorus by Leaching Protein Curd with Saturated Sodium Sulfate Solution

Step in procedure	P removed		
	A	B	C
	per cent	*per cent*	*per cent*
Filtrate from NaOH extraction and acid pptn.	25.7	27.0	25.7
Insoluble residue from NaOH extraction	4.1	8.2	4.1
Na_2SO_4 leachings of curd*	41.4	38.3	57.3
Dialyzed protein (dialyzer ppt.)	15.6	8.7	3.2
Loss by dialysis (by difference)	13.2	17.8	9.7
Total	100.0	100.0	100.0
% P in dialyzed protein	0.32	0.165	0.08

* Leached with saturated Na_2SO_4 at pH 2.2 (Column A), pH 7.0 (Column B), pH 11.5 (Column C).

70 per cent of the meal nitrogen was recovered as protein. The amount of phosphorus removed by the various steps in these experiments is shown in Table VI. Columns A, B, and C contain the results of leaching at pH 2.2, 7.0, and 11.5, respectively. The higher phosphorus content of the protein prepared by leaching the curd with saturated sodium sulfate at pH 2.2 is significant; however, the differences noted at pH 7.0 and 11.5 appear to be more a function of technique than of difference in pH. Preparations obtained by leaching at pH 7.0 contained as little as 0.05 per cent phosphorus if the curd was stirred vigorously during the sodium sulfate extraction.

Vickery (3) extracted the protein from petroleum ether-extracted soy bean meal (Illini) with saturated sodium chloride solution, clarified thoroughly, and dialyzed to obtain a preparation high in nitrogen (16.9 per cent) and low in ash. His experiment was repeated during this study and a product containing only 0.12 per cent phosphorus was obtained, indicatin that sodium chloride in high concentration is effective in removing phytin

Another method (2) often used for preparing soy bean protein is that o extracting the oil-free meal with 5 to 10 per cent saline solution, clarifyin the extract, and dialyzing. The data in Table V do not indicate whethe the removal of phosphorus is attributable to the saline extraction, to th ammonium sulfate precipitation, or to both. To elucidate this point, number of experiments were performed by employing the simplified technique with the hope that the precipitation step with saturated ammoniu sulfate could be eliminated.

These experiments were performed in triplicate and the averages a listed in Table VII. Dialysis was carried out against both distilled wat

TABLE VII

Per Cent of Total Phosphorus and Nitrogen Removed in Various Steps of Extracti Protein with 10 Per Cent Saline and Dialyzing

Step in procedure	Distilled water		Tap water	
	N	P	N	P
	per cent	*per cent*	*per cent*	*per cent*
Residue from NaCl extraction*	33.0	20.5	33.1	24.7
Dialyzed protein, pptd. at pH 5.1	53.0	3.4	44.6	32.1
Filtrate from dialyzer	5.5	0.6	9.2	2.1
Loss by dialysis (by difference)	8.4	75.5	13.2	40.8
Total	99.9	100.0	100.1	99.7
NaCl extract from 1st step	66.9	79.5	67.0	75.0
% dry protein	16.45	0.10	15.75	1.00

* Single extraction with 20 parts of 10 per cent NaCl.

and tap water as indicated. With the exception of pilot plant preparatio (Tables I and III), distilled water was used throughout previous expe ments. As shown in Table VII, a preparation obtained by dialyzi against tap water contained 1.00 per cent phosphorus, while that obtain by employing distilled water contained 0.10 per cent phosphorus. Al it should be noted that the removal of phosphorus occurred during dial sis of the saline extract of the protein.

The failure to remove phosphorus in dialyzing against tap water is tributed to the presence of divalent ions which react with phytin to ren it insoluble. In this connection, it was observed that both phytic acid a sodium phytate readily passed through the cellophane casing used dialysis, although calcium phytate did not. It was also noted that calci phytate was not rendered soluble by dialyzing against 10 per cent sodi chloride.

In further experiments, protein was extracted from the oil-free meal with 1 N sodium chloride solution. The clarified extract was then dialyzed against 1 N sodium chloride solution for 48 hours, followed by dialyzing 5 days against distilled water at 5°. The protein was precipitated from the dialyzer contents by adjusting the pH to 5.1. This preparation contained an average of 0.05 per cent phosphorus.

Dialyzing an alkaline extract against salt solutions gave slightly different results. The alkaline extract was obtained by dispersing the protein with 0.1 per cent sodium hydroxide solution. The combined extracts were clarified by centrifuging at 2000 × g, followed by filtering on a Büchner funnel. This gave a clear amber dispersion which was dialyzed 48 hours against 8 volumes of 1 N sodium chloride solution and centrifuged to remove a slimy precipitate that had formed. This precipitate contained 2.87 per cent phosphorus and 5.26 per cent nitrogen. Salt was then removed from the extract by dialyzing at 5° for 9 days against frequent changes of distilled water. The pH was adjusted to 5.1 and the protein removed by centrifugation. The dry protein contained 0.12 per cent phosphorus and 15.69 per cent nitrogen.

TABLE VIII

Total Nitrogen Found in Various Steps by Removing Phosphorus Prior to Extracting Protein

Step in procedure	Extraction with saturated solution of		
	$(NH_4)_2SO_4$	Na_2SO_4	$MgSO_4$
% N in filtrate (leachings)	0.81	21.59	54.48
% " lost on dialyzing leached meal*	14.11	18.74	17.89
% " in residue after alkali extraction	2.50	2.66	2.19
% " " whey after removal of protein	14.50	12.83	5.84
% " recovered as protein	68.08	44.18	19.60
% P in recovered protein	0.11	0.12	0.30

* By difference.

In another set of experiments, the possibility of removing phosphorus from hexane-extracted flakes, prior to extracting the protein, was investigated. The procedure was as follows: The flakes were extracted with saturated ammonium sulfate, sodium sulfate, or magnesium sulfate. The extracted meal was then dialyzed at 5° against distilled water until free of sulfate ions. The dialyzer contents were adjusted to 0.1 per cent sodium hydroxide to peptize the protein and centrifuged to clarify. Two more alkali extractions were made to insure removal of the protein. The protein was precipitated quantitatively by adjusting the pH to 5.1. The results are listed in Table VIII. It is obvious that ammonium sulfate is

much more efficient in precipitating the protein from the meal than eithe
sodium or magnesium sulfate. The phosphorus content of protein ob
tained from the meal which had been extracted with saturated magnesiur
sulfate was lower than expected, based on previous experience with ta
water. The isolated protein obtained from the meal that was extracte
with ammonium sulfate contained appreciable phosphorus (0.10 per cent
Past experience would indicate that, if the flakes were ground to a flou
prior to removal of the phytin, a more efficient extraction would resul

DISCUSSION

It has been shown that the phytase present in meal extracts liberat
inorganic phosphorus over a pH range of 2.5 to 6.0 (1). The enzymat
liberation of phosphorus becomes especially important when protein is is
lated by dialysis because of the extended time during which the enzym
may act. However, the evidence indicates that the removal of phosphor
by phytase is a minor factor. Dialysis of saline extracts against tap wat
repeatedly failed to remove phosphorus in quantities comparable to th
removed when the extracts were dialyzed against distilled water. It
however, possible that inorganic phosphorus was liberated and render
insoluble by the calcium in tap water. The fact that a saturated soluti
of ammonium sulfate was effective in extracting phosphorus from the w
protein curd or from the oil-free meal, while distilled water was not, wou
appear to exclude the enzymatic liberation of phosphorus as a major fact

Phytin appears to be associated with native protein by a salt-labile lin
age. When the protein-phytin complex is dissociated, it is possible to
move the phytin by dialysis.

Phytin is removed from the wet protein curd, obtained by alkali extra
tion and acid precipitation, by slurrying with a saturated solution of sodiu
or ammonium sulfate. In this case the protein is not dispersed, yet t
phytin is removed. In an experiment in which protein was dispersed
pH 8 in 100 ml. of water containing 100 gm. of ammonium nitrate a
dialyzed against tap water, no phosphorus was removed.

A thorough study of the properties of phosphorus-free protein was
made. However, during the course of the investigation certain obs
vations were noted. Alkaline dispersions of the phosphorus-free prot
appeared to exhibit greater clarity. Plastic disks molded from this pro
uct failed to exhibit the cloudiness noted in ordinary soy bean prot
disks. After dialyzing a sodium chloride extract of meal against distil
water, only 30 per cent of the protein precipitated in the dialyzer (pH 6
and the remainder (excepting the soluble albumin fraction) was prec
tated by adjusting the pH to 5.1. When tap water was used, 90 per c
of the globulin fraction precipitated in the dialyzer. Also, when phy
was removed from the meal prior to alkali extraction, the protein

precipitated quantitatively from the alkaline extract by adjusting the pH to 5.1. Ordinarily it is necessary to adjust the pH to 4.2 before the protein will be precipitated quantitatively. It is indicated, therefore, that both the pH of minimum solubility and possibly the micellar size of the protein are altered by removing the phosphorus.

Phytin slowly dissociates from soy bean protein in alkaline dispersions at pH 11.0 to 11.5 and may be removed by centrifugation. The precipitate contains phytin, together with protein and carbohydrate material. The presence of calcium or barium hydroxide in such dispersions appears to facilitate precipitation. Therefore, a commercial method of preparing soy bean protein of low phosphorus content is indicated.

For fundamental studies, protein is ordinarily extracted with "mild alkali" (pH 8.0 to 9.0) to minimize the amount of degradation. When precipitated with acid, such a preparation may be expected to contain the maximum amount of phosphorus (approximately 1.0 per cent). Phytin was removed from such extracts by dialyzing against 1 N sodium chloride solution.

SUMMARY

The phosphorus content of soy bean protein is shown to be dependent on the method of preparation. Protein was prepared substantially free from phosphorus by methods that would not be expected to degrade or alter the protein.

Substantially all of the organic phosphorus contained in soy bean protein obtained by alkali extraction and acid precipitation behaved as if it were phytin phosphorus and was removed by dialyzing the wet curd against 1 N sodium chloride solution or by extraction with a saturated solution of ammonium sulfate, followed by dialysis to remove the salt.

Phosphorus-free soy bean protein, suitable for fundamental studies, was prepared either by removing the phytin from the oil-free meal prior to extracting the protein or by dialyzing the protein extract against 1 N sodium chloride.

The presence of divalent ions inhibited removal of phytin by dialysis or leaching.

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THE SYNTHESIS OF TRICARBOXYLIC ACIDS BY CARBON DIOXIDE FIXATION IN PARSLEY ROOT PREPARATIONS*

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The presence of oxalosuccinic carboxylase and isocitric dehydrogenase has been reported in pig heart and pigeon liver preparations (1–4). These enzymes have been shown to be involved in the over-all reaction (*c*), which is the sum of the two reactions (*a*) and (*b*).

$$\text{(a)}\qquad \alpha\text{-Ketoglutarate}^{=} + CO_2 \rightleftharpoons \text{oxalosuccinate}^{\equiv} + H^+$$

$$\text{(b)}\qquad \text{Oxalosuccinate}^{\equiv} + TPN^{1}_{\text{red.}} + H^+ \rightleftharpoons \text{isocitrate}^{\equiv} + TPN_{\text{ox.}}$$

$$\text{(c)}\qquad \alpha\text{-Ketoglutarate} + CO_2 + TPN_{\text{red.}} \rightleftharpoons \text{isocitrate} + TPN_{\text{ox.}}$$

These reactions have been studied in animal tissues by spectrophotometric and chemical means (1–4) as well as by techniques involving the use of radioactive carbon (5).

The biochemical significance of tricarboxylic acid formation by this route and the historical development of the subject have been discussed by Ochoa (3), who has also provided detailed information about these reactions as they are catalyzed by an enzyme preparation obtained from pig heart.

We have previously reported the presence in parsley root of similar enzyme systems (6), and the evidence for this conclusion will be presented in this communication.

EXPERIMENTAL

Materials and Methods

The enzyme material used in these studies was a lyophilized sample of a partially purified parsley root preparation (7). This preparation was obtained by treating expressed parsley root juice alternately with acetate buffer at pH 5.0 for several hours and then with dialysis against 0.025 M phosphate buffer at pH 7.4 for 24 hours. Inactive precipitates formed during the course of these treatments and were discarded. The dialyzed preparation was lyophilized. This lyophilized material was shown to contain oxalacetic carboxylase, malic dehydrogenase, oxalosuccinic car-

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[1] Triphosphopyridine nucleotide.

boxylase, and isocitric dehydrogenase. On the other hand, aconitase and fumarase were absent.

Aconitase was prepared by the method described by Ochoa (3). Pigeon liver enzyme was a dialyzed extract of acetone-dried pigeon liver powder described by Evans *et al.* (8). The α-ketoglutaric acid was prepared according to Neuberg and Ringer (9). A sample of barium oxalosuccinate was generously provided by Dr. S. Ochoa. It was prepared for use by the procedure described by him (1). Adenosine triphosphate (ATP) was furnished by Dr. J. Speck. TPN was prepared from beef liver by a modification of the method of Warburg *et al.* (10). Various preparations ranging in purity from 18 to 78 per cent were employed. The purity was calculated after reduction of the TPN by *Zwischenferment* and glucose-6-phosphate from the equation (11):

$$\beta = \frac{1}{c \cdot d} \ln \frac{I_0}{I} = 1.3 \times 10^7 \text{ sq. cm. per mole}$$

DL-Isocitric acid was prepared by the method of Fittig and Miller (12–15). One sample, employed in the first experiments, was provided by Dr. S. Grisolia. It consisted almost entirely of the lactone, and was hydrolyzed before use. A second sample, used in the later experiments, was almost free of the lactone. This sample, which was free of citric acid, was tested for *d*-isocitrate in two ways, (*a*) by its conversion in the presence of aconitase to citrate which was then determined colorimetrically (16), and (*b*) by its oxidation with pyruvate in the presence of pigeon liver enzyme, Mn^{++}, and TPN (17), whereby 1 mole of CO_2 is obtained for each mole of isocitrate present. By both of these methods it was found that 45 to 50 per cent of the material was biologically active; *i.e.*, was presumably *d*-isocitric acid.

Standard Warburg manometric techniques were employed. The procedures used with C^{14} have previously been described (5). Spectrophotometric measurements were made with a Beckman model DU instrument.

Decarboxylation of Oxalosuccinic Acid

Experimental evidence for the catalysis of the decarboxylation of oxalosuccinic acid (OSA) by a heat-labile enzyme found in parsley root has already been published (6), and the dependence of the enzyme on a divalent cation such as Mn^{++} has been shown. The experimental conditions employed in these studies were very similar to those described for the assay of oxalacetic (OAA) carboxylase in the same type of preparation (7), except that lower temperature and lower Mn^{++} concentrations (20° instead of 30°

and 5×10^{-4} M Mn^{++} in place of 1×10^{-2} M Mn^{++}) were used in the studies involving OSA, in order to decrease the value of the blank; in addition, an atmosphere of N_2 was employed in place of air. The decarboxylation of OSA shows first order kinetics with respect to substrate, just as is exhibited by OAA carboxylase of plants (7). The net first order reaction rate constant per mg. of protein, k per mg., when calculated as described for OAA carboxylase,

$$k \text{ per mg.} = \frac{2.3}{(t_2 - t_1)\text{ mg.}} \times \log \frac{\text{OSA}_1}{\text{OSA}_2} - k \text{ of blank}$$

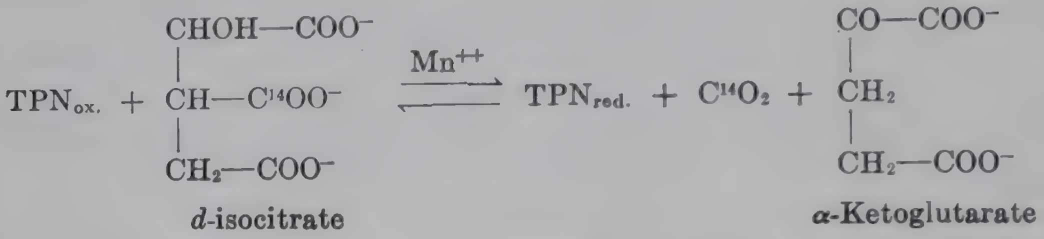
was found to be 0.113 min.^{-1} mg.^{-1} in the experiment already described (6). This value may be regarded as typical of several such values obtained with similar parsley root preparations. One cannot properly compare the reaction rate constants for OSA carboxylase with those obtained for OAA carboxylase, because of the different conditions employed in the two test systems. It is interesting that both OSA and OAA carboxylases from plant sources exhibit the first order kinetics, whereas the corresponding enzymes from animal sources apparently do not (2, 18).

Finally, it is worth noting that it was found necessary to work in the absence of O_2 in order to demonstrate the enzyme. Even in the blank determination (heated enzyme plus Mn^{++} or Mn^{++} alone) appreciable O_2 consumption occurred in an atmosphere of air. This O_2 uptake was greatly accelerated in the presence of active enzyme. This fact is of considerable practical importance, since the O_2 consumption is sometimes sufficient to obscure, almost completely, the CO_2 evolution. Whether it is of any physiological significance appears doubtful, since no O_2 consumption was observed in the presence of isocitrate, TPN, Mn^{++}, and enzyme. The oxidative decarboxylation of OSA follows an S-shaped curve and appears similar to the oxidations previously observed with OAA and Mn^{++} in the presence of metmyoglobin (19).

Exchange of $C^{14}O_2$ with β-Carboxyl Carbon of Isocitrate

The exchange experiments were conducted in the manner previously employed with pigeon liver preparations (5), except for a modification of the procedure used to localize the fixed isotope.

In these experiments, a mixture of TPN, isocitrate, α-ketoglutarate, Mn^{++}, and enzyme was incubated in the presence of $NaHC^{14}O_3$. Since only relatively small amounts of TPN were used, virtually no net reaction took place. The C^{14} initially introduced as bicarbonate did, however, exchange with the β-carboxyl carbon of the isocitrate, indicating the over-all reversibility of the reaction,



$$\mathrm{TPN_{ox.}} + \begin{array}{l}\mathrm{CHOH{-}COO^-}\\ | \\ \mathrm{CH{-}C^{14}OO^-}\\ | \\ \mathrm{CH_2{-}COO^-}\end{array} \overset{\mathrm{Mn^{++}}}{\rightleftarrows} \mathrm{TPN_{red.}} + \mathrm{C^{14}O_2} + \begin{array}{l}\mathrm{CO{-}COO^-}\\ | \\ \mathrm{CH_2}\\ | \\ \mathrm{CH_2{-}COO^-}\end{array}$$

d-isocitrate α-Ketoglutarate

As previously described, an approximate indication of the amount of fixation which occurs may be obtained by evaporation of an ether extract of the acidified reaction mixture to a dry film and determination of its radioactivity. Such a procedure was employed to show that no fixation of CO_2 in organic form occurred in the absence of the substrates isocitrate and α-ketoglutarate, and that the presence of added TPN was likewise essential. ATP alone could not substitute for TPN, but ATP in addition to TPN effected an increase in the exchange reaction above that which occurred with TPN alone (6).

These cofactor effects have been obtained consistently. The most plausible explanation for the effect of ATP appears to be that it prevents in some way the destruction of TPN which occurred in this enzyme preparation. It was determined that immediately after addition of TPN to the enzyme system, during the equilibration period before zero time, more than half of the added TPN disappeared. During the 3 hour incubation period about two-thirds of the TPN present at zero time was lost.[2]

The localization of the fixed isotopic carbon was accomplished by treating the ether extract of the acidified radioactive reaction mixture mentioned above with pigeon liver extract, pyruvate, Mn^{++}, and TPN under conditions known to effect the complete decarboxylation of isocitrate by the reaction,

$$d\text{-Isocitrate} + \text{pyruvate} \rightarrow \alpha\text{-ketoglutarate} + CO_2 + \text{lactate}$$

The CO_2 released by this reaction was radioactive, but not all of the radioactivity resided in this fraction. Moreover it was found that the ether extracts used in these experiments possessed only about one-sixth of the isocitrate originally added. The radioactivity not released as CO_2 by this reaction, therefore, may be present in some product of isocitrate which is apparently formed during the incubation period. The nature of this product has not yet been determined.

It was also shown that use of the pigeon liver enzyme in these experiments did not result in any significant incorporation of C^{14} into the pyruvate added.

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[2] We are indebted to Mr. Eric Conn for these determinations.

TABLE I

$C^{14}O_2$ *Fixation in Parsley Root Enzyme System*

Sample	Dry weight per area*	Amount of radioactivity	
		Net†	Corrected‡
	mg. per sq. cm.	*counts per min.*	*counts per min.*
A. Original parsley root enzyme system deproteinized after 3 hrs. incubation (0.2 ml. sample of 36 ml. total volume)	1.87	82.6	7,450
B. Ether-soluble fraction of A (0.4 ml. sample of 10 ml. total volume)	5.55	497.3	10,590
C. Ether-insoluble fraction of A (2 ml. sample of 10 ml. total volume)	178	0	0
D. Pigeon liver system containing B, deproteinized after 2.75 hrs. incubation (1 ml. sample of 70 ml. total volume)	4.75	100.5	5,440
E. Ether-soluble fraction of D (0.5 ml. sample of 26 ml. total volume)	4.72	127.3	5,090
F. Ether-insoluble fraction of D (1 ml. sample of 60 ml. total volume)	20.5	0	0

* Calculated by dividing the observed dry weight of the sample by 3.47, the area in sq. cm. of the cups used for counting purposes.

† Observed count minus the background count which was generally in the vicinity of 40 counts per minute.

‡ Corrected for thick sample count and then multiplied by the factor $W/(IC \times 197)$ (see "Procedure") so that the resulting values are equivalent to thick sample counts of $BaCO_3$ prepared from the β-carboxyl group of the isocitrate present.

TABLE II

$C^{14}O_2$ *Fixation in Isocitric Acid*

Sample	Amount of CO_2	Dry weight of $BaCO_3$ per area	Amount of radioactivity		
			Net	Corrected*	Relative
	micro-liters	*mg. per sq. cm.*	*counts per min.*	*counts per min.*	*counts*
A. Total CO_2 of original parsley root system at end of 3 hrs. incubation (as $BaCO_3$)	2020	5.6	7720.5	125,000	100
B. β-Carboxyl group of isocitric acid isolated by treatment with pigeon liver system (as $BaCO_3$)	940†	8.7	520.4	8,400	6.8
C. Pyruvate carboxyl C obtained from pigeon liver system by yeast carboxylase (as $BaCO_3$)	7600	7.0	4.0	4.5	

* Corrected both for thick sample count and for dilution by the non-radioactive carbonate added as a carrier.

† 3000 microliters of *d*-isocitrate were calculated to have been originally added to the parsley root enzyme system.

The experimental results and details of the procedures of a typical experiment, one of four which have been completed, are compiled in Tables I and II. In Table I are shown the approximate amounts of radioactivity found in the various fractions. These were determined by simply evaporating an aliquot of a sample to a dry film and then measuring the radioactivity of the film. In Table II are given the accurate determinations of radioactivity initially present as CO_2 in the enzyme system and that later found in the β-carboxyl group of isocitric acid.

Spectrophotometric Evidence for Reduction of TPN by Isocitrate and Its Reoxidation by CO_2 and α-Ketoglutarate

The reversibility of the reaction,

$$\text{Isocitrate} + \text{TPN}_{\text{ox.}} \rightleftharpoons \alpha\text{-ketoglutarate} + CO_2 + \text{TPN}_{\text{red.}}$$

can readily be demonstrated by the use of the spectrophotometric procedures employed by Ochoa (3) in studying the isocitric dehydrogenase of heart muscle. On addition of isocitrate to oxidized TPN in the absence of divalent cations, a partial reduction of the pyridine nucleotide occurs and may be measured spectrophotometrically by determining log I_0/I at 340 mμ. If Mn^{++} is added, the reduction proceeds virtually to completion in the presence of 2×10^{-4} M isocitrate. The reduced TPN can then be reoxidized by addition of α-ketoglutarate and CO_2. Moreover we have found that Co^{++} can be substituted for Mn^{++} in this system. Fig. 1 shows the results of such an experiment conducted in the presence of Co^{++} and the same parsley root enzyme preparation as was employed in the C^{14} experiments.

Although the reactions in the presence of Co^{++} are somewhat slower than those observed with an equivalent amount of Mn^{++}, we have found the former preferable for use in spectrophotometric tests, since there is less tendency for the development of slight turbidities which frequently occur when Mn^{++} is employed. These turbidities have sometimes developed in the presence of Mn^{++} even after removal of phosphate from the enzyme by prolonged dialysis.

We have not attempted to make an accurate measurement of the equilibrium constants of the reactions. However, since Ochoa (3) has determined the constants for the reaction catalyzed by heart muscle, it appeared of interest to see whether such rough data as were available from our experiments would be in approximate agreement with his figures. This has proved to be the case. As an example, K may be calculated from the data of the experiment shown in Fig. 1, the equilibrium reached after 60 minutes being employed. At this point, the ratio $\text{TPN}_{\text{ox.}}:\text{TPN}_{\text{red.}}$ (as determined from the total amount of TPN added, and from the value of log I_0/I at the equilibrium point) = 0.5. The ratio of d-isocitrate to α-ketoglutarate (determined from total α-ketoglutarate added, total isocitrate added, and isocitrate oxidized) = 1.2×10^{-2}. These figures are reasonably accurate. However, in the absence of controlled CO_2 tension, ($CO_2 + H_2CO_3$) can only be roughly estimated. Such an estimate made on the basis of the $CO_2 + H_2CO_3$ in the added bicarbonate gave a value of 3×10^{-3} mole per liter.

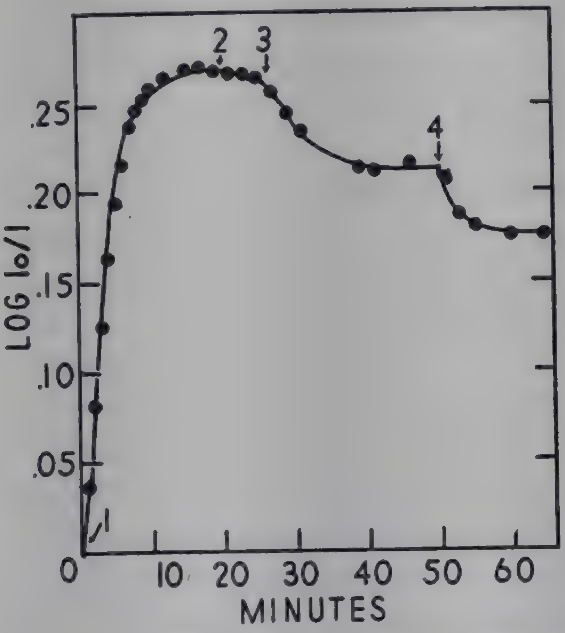

FIG. 1. Reversal of isocitric dehydrogenase from parsley root. Reduction and oxidation of TPN determined spectrophotometrically at 340 mμ. Corex cells, diameter = 1.0 cm. Observations made against a blank which received all additions except TPN. Optical density corrected for changes on dilution. 1.0 ml. of 0.1 M glycylglycine of pH 7.4, 0.1 ml. of 0.01 M cobalt acetate, 0.1 ml. of parsley root enzyme (same solution as that employed in the C^{14} experiment), 106 γ of TPN, made up to a volume of 2.9 ml. with H_2O. At zero time, 0.1 ml. of 0.007 M *d*-isocitrate was added; at *2* 0.1 ml. of 0.5 M α-ketoglutarate; at *3*, 0.1 ml. of 1 M $NaHCO_3$ saturated with CO_2; at *4*, 0.2 ml. of 1 M $NaHCO_3$ saturated with CO_2.

$$K = \frac{(\text{TPN}_{\text{ox.}})(\text{isocitrate})}{(\text{TPN}_{\text{red.}})(\alpha\text{-ketoglutarate})(CO_2)} = \frac{0.5 \times 1.2 \times 10^{-2}}{3 \times 10^{-3}} = 2$$

Ochoa obtained an average of 1.3 for this figure (3). (Ochoa's published result must be multiplied by 10^4 if the units of K are to be reciprocal moles per liter.) In view of the inaccuracies of our determination due to lack of control of temperature, CO_2 tension, and pH, we regard this as reasonable agreement.

Procedure

The enzyme reaction mixture contained the following components in a total volume of 30 ml.: 3×10^{-2} M α-ketoglutarate, 10^{-2} M DL-isocitrate,

2×10^{-2} M phosphate buffer of pH 6.7, 2.7×10^{-3} M $MnCl_2$, 2×10^{-4} M ATP, 9×10^{-5} M TPN, and 95 mg. of enzyme preparation. $C^{14}O_2$ was added last as a neutralized solution of $NaHC^{14}O_3$. The bicarbonate concentration was calculated to be 10^{-3} M, but varied considerably at the end of the incubation period. Incubation was conducted in a closed system at 30° for 3 hours. Appropriate controls were also run. Aliquot samples were removed at zero time for isocitrate and TPN analyses.

At the end of the incubation period, the sample was introduced, via a dropping funnel, into 10 ml. of 10 per cent metaphosphoric acid, and the system was flushed with CO_2-free air into 4 ml. of 1 N CO_2-free NaOH for 15 minutes. The total amount of CO_2 thus collected was determined manometrically from an aliquot of the solution. To another aliquot a weighed amount of non-radioactive Na_2CO_3 was added to insure the collection of an adequate amount of $BaHCO_3$ for a thick sample count. This aliquot, diluted with added Na_2CO_3, was placed in a centrifuge tube and diluted to approximately 10 ml. with water. The CO_2 was then precipitated by the addition of 1 ml. of 10 per cent $BaCl_2$. The $BaCO_3$ was repeatedly washed with water and separated by centrifugation until the supernatant fluid was free of base. The precipitate was then washed with alcohol, transferred to an aluminum cup, and dried for counting purposes. All results on the radioactivity of $BaCO_3$ samples were corrected for dilution by the inactive carbonate which had been added.

The acidified solution from which the $C^{14}O_2$ had been removed was flushed with normal CO_2 gas for 5 minutes to insure complete removal of all traces of $C^{14}O_2$ still remaining. To prevent foaming during this process, a drop of caprylic alcohol was added to the mixture. The mixture was then centrifuged to remove the precipitated proteins. The supernatant fluid contained all of the fixed radioactive C^{14}.

A small aliquot of the supernatant fluid was evaporated to dryness and its radioactivity was counted. The remainder of the supernatant fluid was extracted continuously with ether in a Kutscher-Steudel extractor until all the organic acids present were extracted. It should be mentioned that 12 to 24 hours of extraction are needed for complete removal of the tricarboxylic acids from the aqueous phase. Moreover, it was usually necessary to reextract the first ether extract (after distillation of the ether and dilution and acidification of the aqueous residue) with ether again in order to remove contaminating salts and organic matter which had been carried over as an emulsion during the first extraction. Finally it should be mentioned that the solutions being extracted were kept cold during the extraction procedure by placing the extractors in Dewar flasks filled with ice water. This was done to reduce the transfer of water into the ether extracts during the long period of extraction.

All of the fixed C^{14} was present in the ether extract. The ether was removed by distillation, and the aqueous residue was neutralized and diluted to 10 ml. Aliquots were taken for the various analyses, and one aliquot was evaporated to dryness and its radioactivity determined. Radioactivity values, reported for such dried samples, were thick sample counts calculated in such a way that they would be equivalent to thick sample counts of $BaCO_3$ prepared from the β-carboxyl carbon of the isocitrate present. These values were obtained by multiplying the thick sample counts observed by the factor $W/(IC \times 197)$, where W = total weight of the dried sample being counted, IC = moles of isocitrate calculated to be present in W (from the amount initially added, assuming no destruction), and 197 = molecular weight of $BaCO_3$. Such radioactivity values are not as accurate as those obtained with $BaCO_3$ samples, but do serve as a reasonable approximation of the radioactivity present.

The amount of isocitrate present in the ether extract was determined both by conversion to citrate in the presence of aconitase and the colorimetric determination of the citrate formed, and by oxidation with pyruvate in the presence of pigeon liver enzyme, as previously noted. By this latter procedure, the β-carboxyl carbon of isocitrate is selectively removed. For this determination, 5 ml. of the ether extract (one-half of the total volume) were treated with 10 ml. of pigeon liver enzyme preparation, 20 ml. of 0.5 M acetate buffer of pH 5.0, 2.0 ml. of 0.1 M $MnCl_2$, 1.0 ml. of DPN[3] solution (3 mg. of Schwarz DPN per ml.), 2.0 ml. of TPN (260 γ), and 1.0 ml. of 0.2 M pyruvate. The reaction mixture was incubated in a closed system for 2.75 hours at 30°. At the end of this time, when the evolution of CO_2 was complete, as indicated in a small pilot run in which CO_2 production was determined manometrically, the CO_2 was collected and analyzed for amount and radioactivity, as previously described. To insure adequate acidification of the reaction mixture for CO_2 collection, 0.2 ml. of 2 N H_2SO_4 was added at the end of the incubation period. Since there was some evolution of CO_2 from the pigeon liver enzyme in the absence of added isocitrate, the amount of this blank was also determined manometrically, and appropriate corrections were applied in the calculation of radioactivity of CO_2 actually obtained from the β-carboxyl group of isocitrate.

After this enzymic decarboxylation of the isocitrate and the collection of the liberated CO_2, the reaction mixture was heated in a boiling water bath for 5 minutes and was then centrifuged. The resulting acid filtrate was neutralized and the small additional amount of precipitate which formed was removed by centrifugation. The neutral, deproteinized filtrate was found to contain radioactivity which was completely removed by 24 hours of continuous extraction with ether in Kutscher-Steudel extractors.

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[3] Diphosphopyridine nucleotide.

An aliquot of this ether extract (after removal of the ether by distillation and dilution of the residue with water) was treated with yeast carboxylase. The CO_2 which was liberated from pyruvate by this treatment was collected and tested for radioactivity.

DISCUSSION

In the absence of detailed information about the possible occurrence in plant tissues of a tricarboxylic acid cycle similar to that of animal tissues, it seems necessary to hold in abeyance any definite conclusion in regard to the precise function in plant tissues of CO_2 fixation by β-carboxylations. The results obtained prove, however, that a plant preparation can form tricarboxylic acids by the addition of CO_2 to α-ketoglutarate. The generation of reduced TPN by any suitable hydrogen donor should enable the process to proceed to effect the accumulation of considerable amounts of isocitrate (and citrate, when aconitase is present). It is worthy of note that the equilibrium constant of the over-all reaction (*c*) is not far from unity, indicating that only a small free energy change is involved in the reductive carboxylation.

SUMMARY

The ability of an enzyme preparation from parsley root to catalyze tricarboxylic acid formation by addition of CO_2 to α-ketoglutarate has been demonstrated. A divalent cation such as Mn^{++} or Co^{++} is necessary for the reaction. In the presence of reduced TPN, the product of the initial fixation is reduced to isocitric acid. The over-all reaction is similar to that already studied in animal tissues.

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STUDIES ON KETOSIS IN ALLOXAN-DIABETIC RABBITS

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Animals, such as rabbits (1–3), dogs (4, 5), monkeys (6), and rats (7–9), made diabetic by parenteral injection of alloxan, suffer from hyperglycemia, glycosuria, and hyperlipemia, and the diabetes resembles that produced by pancreatectomy. The alloxan-diabetic animals mentioned above, except rats (7, 8), excrete little or no acetone bodies in the urine, usually live for long periods without receiving any injection of insulin, and die of inanition and not in acidosis and coma. The rats, however, suffer from an early onset of ketosis and die of ketosis and coma within 7 days after the injection of alloxan. The longer survival of alloxan-diabetic animals other than rats seems to be related to the lower urinary excretion of acetone bodies by those animals. Thorogood and Zimmermann (5) observed that the severe glycosuria and high insulin requirement in alloxan-diabetic dogs could be markedly reduced by depancreatization. Pancreatectomy, however, produced an immediate onset of ketosis and the animals died of acidosis and in coma. Greeley (10) reports that depancreatized Herbivora, like the rabbit and goat, though resembling depancreatized Carnivora, like the dog and cat, differ from the latter in that there is no ketosis and the animals live in quite healthy conditions for long periods, thus resembling alloxan-diabetic animals. Thorogood and Zimmermann (5) suggested the existence of a second pancreatic hormone that might prevent ketosis and coma.

In the present paper the effects of intravenous injection of alloxan on the urinary excretion of acetone bodies and sugar by rabbits have been investigated. The effects of partial removal of the pancreas on the urinary excretion of acetone bodies and the formation and utilization of acetone bodies by alloxan-diabetic rabbits have also been studied.

EXPERIMENTAL

Healthy Himalayan rabbits were fed germinated gram (*Cicer arietinum*) during the experimental period. The animals were kept in separate metabolism cages and the urine collected under toluene. The total acetone bodies excreted in the 24 hour output of urine were estimated by the method of Behre (11). Each of the rabbits then received a single intravenous injection of alloxan (200 mg. per kilo). Hypoglycemia was prevented in these animals by repeated intravenous injections of glucose. The 24 hour urinary excretion of acetone bodies and sugar was determined for varying periods up to 2 weeks after the injection of alloxan. The results are given in Table I.

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TABLE I

24 Hour Urinary Excretion of Acetone Bodies and Glucose by Rabbits Both before and Several Days after Injection of Alloxan

Rabbit No.		Acetone bodies (mg.) and glucose (gm.) after injection of alloxan											
		0	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	10 days	12 days	14 days
1	A.	1.3	0.6	0.9	18.6	22.4	51.3	7.8	6.0	1.7	2.6	1.3	1.5
	G.	0	0	1.9	3.5	4.2	4.0	4.8	4.6	5.0	4.2	3.5	4.0
2	A.	0.8	0.8	0.6		3.0		25.0	193.6	116.0	13.1	6.0	1.0
	G.	0	0	2.2	2.8	7.3		10.0	10.0	7.6	5.0	6.6	6.2
3	A.	1.0		0.4		12.0		15.5	5.0		1.0	3.2	1.0
	G.	0		1.5	1.4	1.7	1.1	3.0	3.0		3.0	3.5	3.0
4	A.	0.9		0.3		200.0	208.0	Dead					
	G.	0		1.3		1.6	2.2						
5	A.	1.0		5.6		94.0	272.0	"					
	G.	0	1.4	6.0	3.0	3.0	2.8						
6	A.	2.1	2.4	2.1	18.0	77.0		130.0	108.0	71.0	98.0	8.6	1.3
	G.	0	0.7	2.1	5.2	6.0		6.1	4.8	1.5	5.2		4.5
7	A.	0.8	1.0	0.6	56.4	151.6	160.0	35.2	20.0		4.4	1.3	1.2
	G.	0	1.6	3.2	3.0	3.5	3.0	2.7	3.0		3.0	2.5	2.5
8	A.	1.8	1.6	2.3	15.5	38.7	62.6		128.0	43.5	16.5	18.5	16.7
	G.	0	0.8	2.8	5.6	6.0	6.6		6.1	5.3	6.0	6.1	5.0
9	A.	1.1	1.2	2.0	58.0	124.0	Dead						
	G.	0	1.3	4.5	5.5	3.3							
10	A.	1.3	1.3	0.9	8.9	17.0	25.4	35.8	10.2	2.8	1.8	1.5	
	G.	0	2.3	4.6	8.3	7.5	6.6	5.2	5.5	5.0	4.3	5.2	

A. = acetone bodies; G. = glucose.

To determine the effect of removing the pancreas on the urinary excretion of acetone bodies by alloxan-diabetic rabbits, about half of the pancreas was removed from three rabbits under ether anesthesia 8 days after the injection of alloxan. The 24 hour urinary excretion of acetone bodies by these animals, both before and after the removal of the pancreas, is shown in Table II.

In all of the rabbits there was considerable increase in the urinary excretion of acetone bodies for the first few days after the injection of alloxan, which fell to almost a normal level within 2 weeks. In order to find out whether the decreased urinary excretion of acetone bodies was due to increased utilization of acetone bodies by the tissues of the alloxan-diabetic animals, three rabbits were made diabetic by injection of alloxan. When the urinary excretion of acetone bodies was very high, the animals were anesthetized with ether, and blood was collected from the femoral artery and the femoral vein of one hind leg. The total acetone bodies of both arterial and venous blood were estimated by the method of Behre (11), as used in the estimation of acetone bodies in urine. When the urinary excretion of acetone bodies was reduced, both arterial and venous blood were again collected from the other hind leg, and total acetone bodies estimated as before. The results are given in Table III.

TABLE II

24 Hour Urinary Excretion of Acetone Bodies by Rabbits before and after Partial Removal of Pancreas on 8th Day after Injection of Alloxan

Rabbit No.	Acetone bodies after injection of alloxan, mg.											
	0	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	10 days	12 days	14 days
1	0.9	0.8	123.0	1.1	2.5	18.6	165.0	148.0	51.6	15.0	4.0	1.2
2	1.4	0.8	2.2	18.0	22.0		7.0	5.1	17.3	3.6	1.9	1.3
3	2.5	1.8	2.0	2.1	15.6	77.3	150.4	108.0	70.0	73.0	22.5	1.8

TABLE III

Acetone Bodies of Arterial and Venous Blood of Alloxan-Diabetic Rabbits

Rabbit No.	Days after injection of alloxan	Daily urinary excretion of acetone bodies	Acetone bodies, per 100 cc. blood	
			Arterial	Venous
		mg.	*mg.*	*mg.*
1	4	151.6	32.0	31.0
	10	4.4	14.5	13.8
2	7	128.0	27.8	27.1
	14	16.7	15.9	15.3
3	12	1.5	9.1	8.5

DISCUSSION

After the injection of alloxan the rabbits began to excrete increased amounts of acetone bodies in the urine on the 3rd day, a maximum being reached towards the end of the 1st week. Some animals died at this stage in acidosis and coma. The rabbits which outlived this critical period began to excrete lower amounts of acetone bodies, and towards the end of the 2nd week the excretion became normal. These animals lived for about

2 months. There was practically no relation between the urinary excretion of sugar and acetone bodies. The urinary excretion of sugar was still high when the diabetic rabbits began to excrete normal amounts of acetone bodies. Alloxan diabetes is characterized by the specific necrosis of the β-cells of the islets of Langerhans and represents only insulin deficiency in the animals. From the results obtained it appears that the absence of insulin alone is not responsible for all of the symptoms of diabetes. Ketosis, particularly, is not a result of insulin deficiency alone, as can be seen from the fact that when the excretion of ketone bodies became normal the urinary excretion of sugar was still high. Acetonuria was not increased after partial pancreatectomy in the alloxan-diabetic rabbits. The claim of Thorogood and Zimmermann (5) that a second antiketogenic hormone is present in the pancreas, therefore, could not be confirmed. It seems that the factor or factors controlling ketosis lie in some other organ.

The rapid rise in the urinary excretion of acetone bodies for the first few days after the injection of alloxan indicates that there is increased ketogenesis, possibly in the liver. The fall in the urinary excretion of ketone bodies at the end of the 2nd week may be due to either diminished formation of ketone bodies or increased utilization of ketone bodies. Table III shows that when there is increased excretion of acetone bodies the acetone body content of both arterial and venous blood is high, indicating that there is ketosis, and tissues of the alloxan-diabetic rabbits cannot utilize ketone bodies. The secondary lowering of ketone bodies is associated with lower blood acetone bodies. Here again there is no difference in the acetone body content of arterial and venous blood. The claim of Greeley (10) that Herbivora do not excrete acetone bodies is, therefore, not tenable in the case of rabbits. Alloxan-diabetic rabbits show ketosis and ketonuria at the onset of diabetes, which are, however, not permanent.

SUMMARY

Diabetes produced in rabbits by the intravenous injection of alloxan is not free from ketosis. There is an early onset of acetonuria which reaches a maximum towards the end of the 1st week after the injection of alloxan and then falls gradually until normal levels are reached.

Glycosuria has no relation to the acetonuria in alloxan-diabetic rabbits. When the rabbits excrete a fairly normal amount of acetone bodies in the urine, the sugar content of the urine is still high.

The decrease in acetonuria, finally reaching normal levels, is due to a decrease in ketogenesis itself and not to increased utilization of acetone bodies by the tissues.

The partial removal of the pancreas in alloxan-diabetic rabbits does not affect the course of ketosis in the animal.

It is suggested that the absence of insulin alone is not responsible for ketosis in diabetics and that ketosis is probably due to some other extra-pancreatic factor.

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BIOCHEMICAL CHARACTERIZATION OF LYMPHOID TISSUE PROTEINS*

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The possible rôle of the lymphoid tissues as a depot or source of certain blood and tissue proteins has aroused considerable interest in the chemistry and physiology of these structures. The demonstration by White and Dougherty (1) of increased levels of serum β- and γ-globulins accompanying lymphocytic dissolution under conditions of augmented pituitary-adrenal cortical activity suggested that at least a portion of these proteins arose from the lymphocyte. Preliminary studies by these authors of saline extracts of rabbit lymphoid tissue revealed the presence of proteins with electric mobilities similar to those of serum β- and γ-globulin. Normal γ-globulin has also been demonstrated by Kass (2) in human lymphoid tissue. Furthermore, in animals immunized by the intraperitoneal injection of antigen, relatively high titers of antibody were found in saline extracts of lymphoid tissue by Dougherty, Chase, and White (3). The presence of antibody in lymphocytes has been confirmed by Harris *et al.* (4) and by Mondolfo and Hounie (5). This antibody, presumably immune globulin, may be readily released by pituitary-adrenal cortical stimulation *in vivo* (6), and upon incubation of surviving lymphoid tissue *in vitro* (7).

The function of the lymphocyte in the physiology of normal and immune globulins, the contribution of this cell to processes concerned with nitrogen mobilization in circumstances of stress, and the influence of pituitary-adrenal cortical secretion on the rates of these processes afford a partial basis for the elucidation of the rôle of the lymphocyte in the normal and diseased organism (*cf.* (8)). It would seem that detailed knowledge of the chemical constituents of normal and malignant lymphocytes would contribute to a further understanding of this rôle. As an initial approach to this problem, a study has been made of certain characteristics of the proteins which may be extracted from rabbit and rat lymphoid tissue,

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from calf thymus, and from a transplantable lymphosarcoma of the mouse.

EXPERIMENTAL

Efficiency of Extraction of Lymphoid Tissue Proteins with Different Aqueous Solvents

The goal first sought was the most efficient method of extraction of lymphoid tissue in terms of the *total* amount of protein present in the extracts. It was recognized, of course, that the relative proportions of different proteins extracted would vary greatly with the extraction solvent, and that the most efficient method as far as total protein was concerned need not necessarily be the most desirable for any particular protein.

TABLE I

*Comparison of Methods for Extraction of Rabbit Lymphoid Tissue**

Extraction solvent	Total nitrogen		Organic P	N:P
	Per ml. extract	Per gm. tissue		
	mg.	*mg.*	*γ per ml.*	
Water	3.19	8.5	178	17.9
" pH 7.6	4.12	11.9	213	19.3
0.05 M Na_2SO_4	2.06	6.5	85	24.2
0.02 " NaCl	1.51	4.8	115	13.1
0.03 " KOH	1.43	1.9	80	17.9
0.02 " phosphate buffer, pH 7.8	1.70	5.7	92	18.5

* In each instance the ratio of the extraction fluid (ml.) to tissue (gm.) was 4:1.

Pooled rabbit lymphoid tissue (lymph nodes, thymus, and appendix) was obtained fresh from animals killed by the injection of air into the marginal ear vein. The tissue, trimmed of fat and connective tissue, was finely minced and ground for about 15 minutes in a chilled mortar with washed sea sand and approximately twice its weight of extraction fluid. The extract was then subjected to a preliminary centrifugation to remove the sand and most of the tissue débris. Thereafter, the extract was cleared by high speed centrifugation at 15,000 R.P.M. and 5° for 30 minutes in the International refrigerated centrifuge. Total nitrogen analyses and organic phosphorus determinations were performed on the extracts by modifications of the micro-Kjeldahl (9) and the Fiske and Subbarow (10) methods, respectively. The results obtained are detailed in Table I.

Of the various solvents tested, distilled water adjusted to pH 7.6 with NaOH extracted the greatest amount of nitrogen from pooled rabbit lymphoid tissue. An examination of the nitrogen-phosphorus ratios (N:P) in the various extracts revealed that a considerable amount of nucleic acid or nucleoprotein was probably present in each case. As might be expected, the extracts made with NaCl of low ionic strength (0.02 M) contained relatively large amounts of organic phosphorus and were extremely viscous, probably due to the presence of large amounts of nucleic acids. The least amount of phosphorus was found in extracts made with 0.05 M Na_2SO_4; this phenomenon was earlier noted by Halliburton (11).

It is apparent from Table I that even low concentrations of salt markedly inhibited the extraction of nitrogen by aqueous solution. The reduction in efficiency was of the order of 50 per cent with sulfate, chloride, hydroxide, and phosphate. On the other hand, it is equally striking that adjusting the pH of distilled water to 7.6 with NaOH increased the degree of extraction by approximately one-third. The relative efficiency of extraction for the latter solvent appeared to be so much higher than for the other aqueous solvents tested that it was chosen for the remainder of the work. Also of advantage was the fact that, even though fairly large amounts of nucleic acid appeared to be present, the extracts obtained with this solvent were comparatively clear and could be examined electrophoretically without difficulty.

Electrophoretic Examination of Extracts of Lymphoid Tissue from Different Animals

Mildly alkaline extracts were made essentially as described above with lymphoid tissue obtained from each of the following sources: pooled lymphoid tissue from rabbit or rat, transplanted mouse lymphosarcoma, and calf thymus. All the procedures outlined were carried out at temperatures between 0–5°. After high speed centrifugation of the extracts, the supernatant material was dialyzed against two or three changes of 0.1 M sodium diethylbarbiturate at pH 8.6 for a total of 72 to 96 hours. Both total and non-protein nitrogen analyses were made on the protein solution. The amount of protein present (protein nitrogen × 6.25)[1] varied between 2.5 and 3.0 per cent in most instances.

The dialyzed extracts of lymphoid tissue were subjected to electrophoretic analysis in the Tiselius apparatus. All determinations were made in a constant temperature bath held at 4°, with 0.1 M sodium diethylbarbiturate at pH 8.6 ($K = 3.82 \times 10^{-3}$ mho per cm.) as the buffer, and with a constant current of approximately 15 ma. passing through the cell. The patterns obtained when photographs were taken of the moving boundaries were qualitatively quite similar for the four types of lymphoid tissue studied

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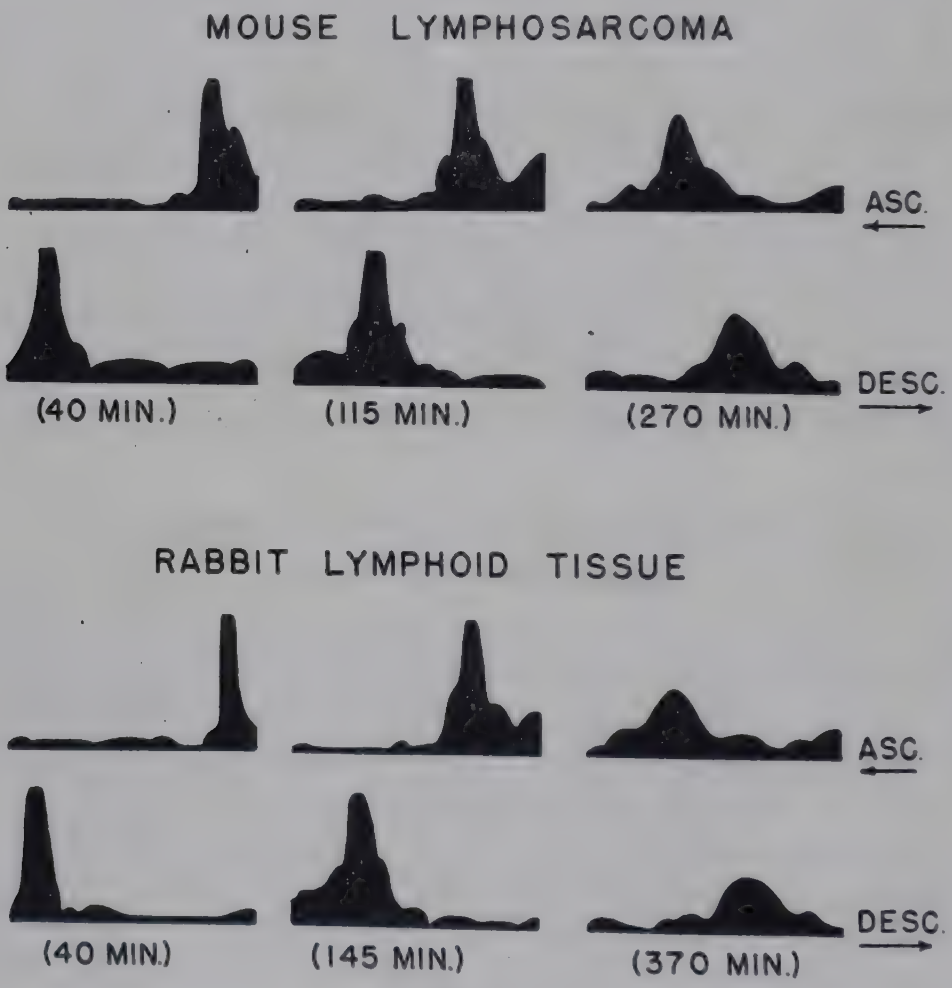
[1] It is recognized that this calculation may not always be acceptable for the estimation of protein. In the present study, however, the values obtained for the quantity of protein present agreed remarkably well, whether calculated as protein nitrogen × 6.25, or determined by actual weighing of the lyophilized material.

(*cf.* Figs. 1 and 2). In all cases, seven components were observed, each possessing a characteristic mobility.

The presence of seven electrophoretically distinct components, each of which moved with a more or less definite electric mobility regardless of the source of the lymphoid tissue, is shown in Table II. It is interesting to note the occurrence of peaks which move with mobilities similar to that of


MOUSE LYMPHOSARCOMA

RABBIT LYMPHOID TISSUE

FIG. 1

serum albumin (Component 2), α_1-globulin (Component 3), α_2-globulin (Component 4), β-globulin (Component 5), and γ-globulin (Component 7). It is not likely, however, that these proteins in lymphoid tissue extracts are identical in all cases with the corresponding serum proteins. The most rapidly moving component (Component 1), and possibly the next most rapid (Component 2), may have been nucleic acid or nucleoprotein (see below).

Extracts of each of the four tissues studied contained two major components, which accounted for 60 to 80 per cent of the total protein extracte (Table II). These two components traveled at electric mobilities of 4.

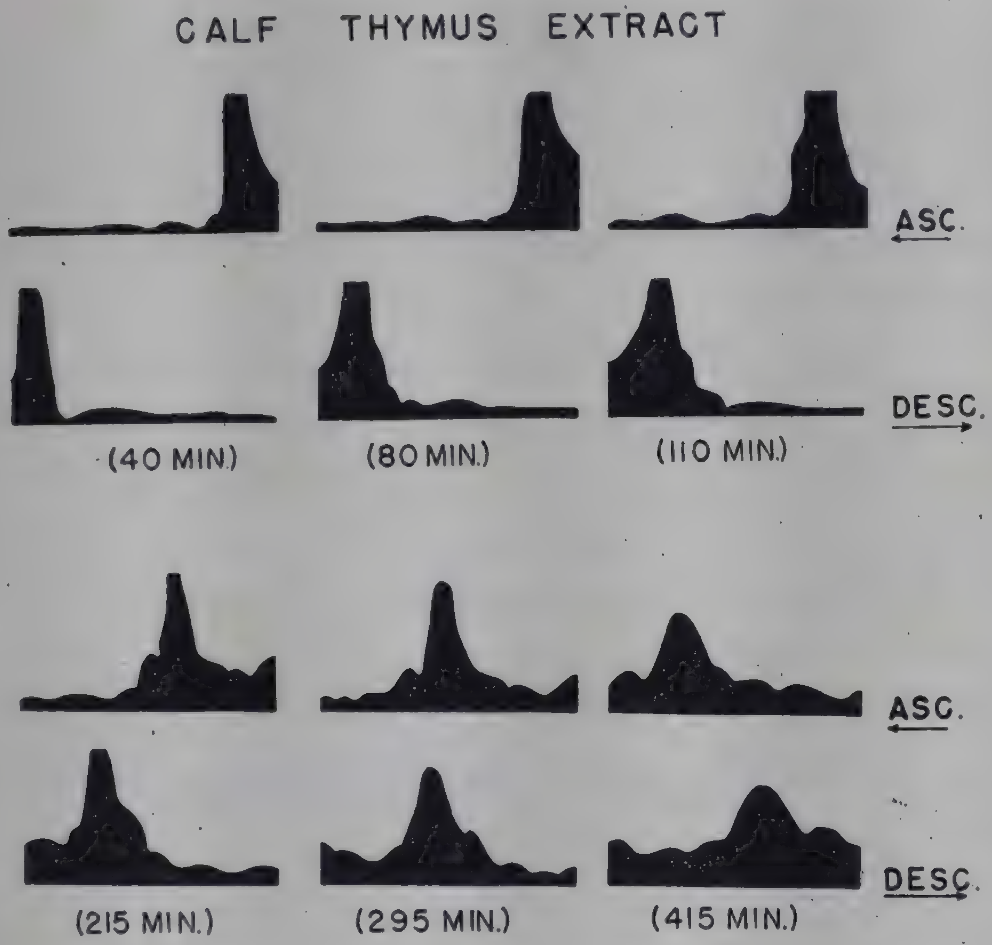


FIG. 2

TABLE II

Electrophoretic Analysis of Lymphoid Tissue Extracts

Component No.	Electric mobility, 1×10^{-5} sq. cm. per volt per sec.				Per cent composition			
	Calf thymus	Rabbit lymphoid tissue	Rat lymphoid tissue	Mouse lymphosarcoma (transplanted)	Calf thymus	Rabbit lymphoid tissue	Rat lymphoid tissue	Mouse lymphosarcoma (transplanted)
1	13.6	14.1	11.5	13.4	3.7	1.0	1.8	0.4
2	6.6	6.4	6.9	7.0	5.0	3.8	3.1	1.4
3	5.0	5.6	5.0	5.1	7.1	6.2	2.1	2.3
4*	4.3	4.0	4.3	4.2	15.0	14.8	9.2	14.2
5*	3.3	3.0	3.8	3.2	46.6	61.1	70.1	66.8
6	2.3	1.6	2.3	2.0	10.7	9.1	8.2	10.0
7	1.0	1.4	1.4	1.1	11.9	4.0	5.5	4.9
Protein in extract, %					2.8	2.7	2.7	2.7

* Major components.

to 4.3 × 10^{-5} (Component 4) and 3.0 to 3.8 × 10^{-5} sq. cm. per volt per second (Component 5), respectively. The distribution of the remaining protein among the other five components was also quite similar in the four extracts. Thus, it appears that there is a quantitative, as well as qualitative, similarity among the protein components of lymphoid tissue, whether the latter is derived from rat or rabbit, from calf thymus, or mouse lymphosarcoma.

Cold Ethanol Fractionation of Calf Thymus Extracts

Frozen calf thymus, the proteins of which appeared to be representative of lymphoid tissue proteins in general, was chosen for fractionation studies, since it was a readily available source material. The methods employed were similar to those developed by Cohn *et al.* (12) for the fractionation of human blood plasma.

TABLE III

Yields and Properties of Cold Ethanol Fractions from Calf Thymus Extract

Fraction	Ethanol concentration at precipitation	Relative yield, per cent of protein in original extract	pH of 0.5 per cent solution in distilled H_2O	N:P
	vol. per cent			
A	0	100.0	7.5	17.0
B	10	19.0	6.5	11.3
C	20	4.6	6.8	27.0
D	30	4.8	6.9	20.4
E	40	36.0	6.6	15.1
F	Supernatant at 40%	10.5	6.3	22.3

Extracts of the frozen tissue were made as already described. All procedures were carried out in the cold room between 0–5°. To 1500 ml. portions of the crude extract was added with continuous stirring a 50 per cent ethanol-water mixture at the rate of 30 ml. per hour. A capillary tube was used for this addition. The materials insoluble at final concentrations of 10 and 20 per cent ethanol were separated by centrifugation at 2000 R.P.M. for 1 hour. The addition of ethanol to the remaining solution was then continued, 95 per cent ethanol being employed. Fractions insoluble at 30 and 40 per cent ethanol were thus obtained, as well as a fraction not precipitated at a final concentration of 40 per cent ethanol. Each of the precipitates obtained by centrifugation was partially freed of ethanol by decanting, redissolved in distilled water, recentrifuged to remove any water-insoluble material, and then lyophilized. The supernatant at 40 per cent ethanol concentration was dialyzed against three changes of slightly alkaline water (pH 7.6) for 3 days, centrifuged to remove substances precipitating during dialysis, and also lyophilized. The yields and certain properties of these fractions are given in Table III. The total yield may be calculated to be 75 per cent of the protein present in the original extract.

Electrophoretic analysis was carried out by dissolving each of the fractions in distilled water in a concentration of 2 per cent, and dialyzing as described earlier against 0.1 м barbiturate buffer. Dialyzed samples after centrifugation were taken for the determination of total and non-protein nitrogen and organic phosphorus. The patterns observed in the Tiselius apparatus are shown in Fig. 3. It may be noted that Fractions B, C, D, and E, representing the precipitates obtained with 10, 20, 30, and 40 per cent ethanol, respectively, each contained three major boundaries. The starting material (Fraction A), as well as the supernatant not precipitating at 40 per cent ethanol (Fraction E), each possessed varying quantities of the original seven components. The percentage composition of each fraction is given in Table IV.

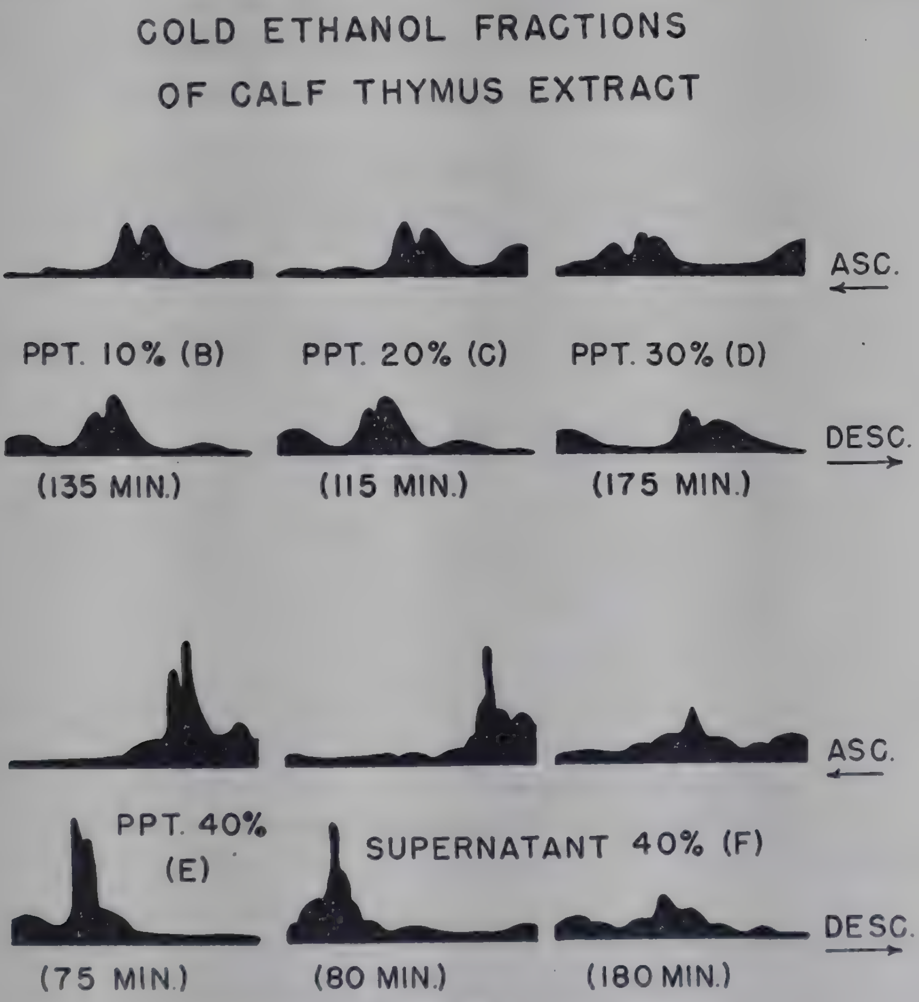


Fig. 3

It is possible that the components in the various fractions shown in

Table IV do not correspond to one another exactly as represented, since changes in mobility of the order of 10 per cent were observed after fractionation. It is well known that the electric mobility of a protein may vary considerably with variations in the types and quantities of other proteins and other substances in the same solution (*cf.* (13)). It will be observed, however, that a partial separation of the seven components present in the original extract of calf thymus has been effected. The relatively large amounts of organic phosphorus in Fractions B and E (low N:P ratios, Table III) would appear to be associated with the presence of the major portions of Components 1 and 2 in these fractions (see Table IV). It may be suggested, therefore, that these two fast components contain large amounts of nucleic acid, and may, indeed, represent the major portion of the nucleoprotein material present in the original extract. The latter statement would seem to be borne out by the large N:P ratios found for Fractions C, D, and F (Table III).

TABLE IV

Electrophoretic Analysis of Cold Ethanol Fractions from Calf Thymus Extract

Component No.	Electric mobility*	Per cent composition					
		Original extract (A)	Ppt. 10 per cent (B)	Ppt. 20 per cent (C)	Ppt. 30 per cent (D)	Ppt. 40 per cent (E)	Supernatant 40 per cent (F)
1	13.6	3.7	8.4	3.5			2.3
2	6.6	5.0				13.8	10.5
3	5.0	7.1			32.9		6.0
4	4.3	15.0	61.0	62.5	32.9	43.8	21.1
5	3.3	46.6	30.6	34.0	34.2	42.4	30.1
6	2.3	10.7					12.8
7	1.0	11.9					17.2
Per cent yield†........		100	19.0	4.6	4.8	36.0	10.5

* 1×10^{-5} sq. cm. per volt per second.

† Per cent of solid material in original extract present in each fraction. Total yield, 74.9 per cent.

Biological Activity of Calf Thymus Extracts in Relation to Lymphoid Tissue Structure and Function

Preliminary observations have been made on the possible biological activity of lymphoid tissue extracts and fractions. Results were obtained which indicated that substances were present in alkaline extracts of calf thymus which were capable of causing acutely an elevation in the blood lymphocyte level of the adult Sprague-Dawley rat. Further investigation revealed that Fraction E, precipitated by 40 per cent ethanol, was most active in this regard; Fraction B, precipitated by 10 per cent ethanol, was next in activity. Thus, the average rise in total lymphocytes 1 hour after the intraperitoneal injection of 10 mg. of Fraction B (in distilled water) into three male rats was of the order of 16 per cent. Fraction E resulted in an elevation of 44.3 per cent under the same conditions. Distilled water alone had little or no effect, and the other fractions tested caused a depression in total lymphocyte count of about 10 to 20 per cent.

TABLE V

Effect of Aqueous Extracts of Calf Thymus on Organ Weights of Adult Rats

The results are averages, ± standard deviations where indicated, and are expressed as mg. per 100 gm. of body weight.

Group	No. of rats	Autopsy body weight	Thymus	Spleen	Liver	Adrenals
		gm.				
Control, ♀	4	156	245 ± 13	508 ± 181	4730	24.9
F4A,* ♀	8	151	282 ± 28	576 ± 99	5028	27.5
Control, ♂	12	241	241 ± 24	366 ± 37	4629	14.6
F7A2,† ♂	6	228	305 ± 36	420 ± 49	4922	15.9
F7B,‡ ♂	6	229	302 ± 35	384 ± 40		15.4
F7E,§ ♂	6	243	304 ± 31	386 ± 56		14.7

* Initial aqueous extract; 10 mg. per day for 8 days.

† Initial aqueous extract; 10 mg. per day for 14 days.

‡ 10 per cent EtOH precipitate; 10 mg. per day for 13 days.

§ 40 per cent EtOH precipitate; 10 mg. per day for 13 days.

In an attempt to ascertain the site of action of the active materials, extracts and fractions of calf thymus were injected daily into adult male rats over a period of 1 to 2 weeks. At the end of this time, the animals were sacrificed, and certain organs weighed (liver, spleen, thymus, adrenals, heart, kidney, testes). As Table V reveals, the most profound changes were found in the thymus, where significant increases in weight were uniformly observed. Histological examination demonstrated the presence of essentially normal thymic structure. Fractions B and E were again relatively active, causing selective enlargement of the thymus, and apparently not affecting the spleen and other organs investigated. It will be recalled that these two fractions were richest in organic phosphorus content. The results suggest that some substance present in calf thymus, possibly nucleoprotein or nucleic acid, may be capable of causing thymic hyperplasia, and of stimulating lymphocyte production or release in the adult rat.

DISCUSSION

The first detailed study of the proteins present in lymphoid tissue was apparently that reported by Halliburton in 1887 (11). In this remarkable investigation, the physical properties of aqueous extracts of cat and dog lymphoid tissue were carefully studied. On the basis of heat coagulability, solubility at different salt concentrations, and viscosity, Halliburton was able to demonstrate the presence of four or five different proteins in the extracts. With considerable foresight, he likened certain of the proteins present to nucleoprotein and to serum globulin.

Mammalian lymphoid tissue, and thymus in particular, has long been recognized as a rich source of "animal" nucleoprotein, because of its predominantly cellular structure. Recently, preliminary investigations on the fractionation of thymus nucleoproteins have been reported (14, 15). Stern (14) has demonstrated the presence of ribose nucleoprotein in the cytoplasm of calf thymus tissue and desoxyribonucleoprotein in the nuclear material. The latter protein has been prepared in an electrophoretically and ultracentrifugally homogeneous form after exhaustive extraction of the cytoplasmic proteins with 0.14 M NaCl. Gjessing (15) found nucleic acid in fractions of the separated stroma, lymphocytes, and saline-soluble components of calf thymus and mouse thymoma. In the present investigation, varying amounts of nucleic acid were obtained with different aqueous solvents. The largest amount was apparently extracted with 0.02 M NaCl. The two fast components observed electrophoretically in aqueous alkaline extracts of lymphoid tissue contained the bulk of this material. A similar conclusion was forthcoming from the work of Abrams and Cohen (16), who found in aqueous extracts of human tonsil tissue a fast component (mobility about 12×10^{-5} sq. cm. per volt per second) which was characterized as free ribonucleic acid. This same material was present in extracts of lymphoid tissue from other sources (Table II).

Halliburton (11) and Stern (14) both pointed out the presence of several globulin-like proteins in aqueous extracts of lymphoid tissue. From the present study, there would appear to be at least five proteins, discounting the two components of rapid mobility as possibly consisting mainly of nucleic acid. Two of these proteins, at least, seem to have certain properties in common with the serum proteins, β- and γ-globulin. A substance with an electric mobility similar to that of serum β-globulin has been found in extracts of rabbit lymphoid tissue (1), calf thymus, rat and rabbit lymphoid tissue, and mouse lymphosarcoma (present study), and human lymphoid tissue (16). However, the latter workers have been unable to demonstrate the presence of a protein with the electric mobility of γ-globulin in aqueous extracts of lymphoid tissue. The reason for this is not clear, but it may be recalled that Kass (2) has noted the presence of normal γ-globulin in lymphoid tissue and that antibody (presumably immune γ-globulin) has been demonstrated in lymphoid tissue by a number of investigators (17, 3–5, 7). Whether or not these globulins are identical with their serum counterparts must, of course, await their isolation and characterization. Both cold ethanol fractionation (present study and (15)) and ammonium sulfate fractionation (16) have been employed in such attempts.

The presence of substances in lymphoid tissue which may have some biological activity has long been suspected. The investigations of Bomskov, purporting to demonstrate the presence of a thymus hormone active in growth and carbohydrate metabolism, have been the object of a critical denial by Andreasen (18). Parsons *et al.* (19) have reported the results of detailed studies on the action of certain pentose nucleotides on normal and tumorous mouse tissue. Of particular interest was the occurrence of a lymphocytosis in mice receiving injections of these substances. In the present investigation, injection of extracts and fractions of calf thymus had a similar effect, and the activity seemed to be concentrated in the fractions rich in nucleic acid or nucleoprotein. The same fractions were potent in producing enlarged thymus glands in rats after repeated injection for 1 to 2 weeks. It seems probable that the increased number of lymphocytes appearing in the blood after injection of these extracts was due to stimulation of the thymus and possibly other lymphoid structures. The mechanism of action of these extracts, whether direct or indirect, is obscure at the present time. The results obtained, however, could be explained by the presence in lymphoid tissue of a substance which has the capacity of depressing the normal pituitary-adrenal cortical mechanism controlling the rate of lymphocyte dissolution and formation (*cf.* (8)). This possibility is being further explored.

SUMMARY

Extraction of rabbit lymphoid tissue with various aqueous solvents revealed that the greatest amount of protein was obtained with mildly alkaline water (pH 7.6). The extracts obtained, although they apparently contained relatively large amounts of nucleic acid, were clear enough to permit electrophoretic analysis.

Examination of alkaline extracts of rabbit and rat lymphoid tissue, calf thymus, and mouse lymphosarcoma in the Tiselius apparatus disclosed the presence of at least seven components in all instances. The electric mobilities and percentage distribution of the various boundaries were quite similar for the four types of extracts studied. Substances possessing the mobility of serum β- and γ-globulin were found to be present.

Fractionation of extracts of calf thymus has been attempted with the cold ethanol technique. Five fractions were obtained, two of which ap-

peared to be rich in nucleoprotein. Intraperitoneal injection of these two fractions resulted in an increase in the blood lymphocytes of adult rats. Repeated injection over a period of 1 to 2 weeks also caused thymic hyperplasia.

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THE INABILITY OF THE RAT TO UTILIZE THE METHYL GROUPS OF METHIONINE SULFONE AND OXIDIZED CASEIN FOR METHYLATION OF HOMOCYSTINE

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Methionine sulfone has been found incapable of substituting for methionine in the growth of the rat (1). When casein is oxidized by hydrogen peroxide, the resulting oxidized casein (2) cannot support the growth of the rats unless the tryptophan and methionine oxidized in the process are replaced (3). This fact, together with chemical evidence (2), indicates that in the modified protein molecule methionine has been converted to the sulfone, rather than to the sulfoxide which will support growth (4). By paper chromatography of hydrolysates, Dent (5) recently confirmed the absence of methionine sulfoxide and the presence of the sulfone in oxidized casein.

The rat experiments (1, 3), as well as the frequent use of oxidized casein in bacteriological assay work, indicate the absence of metabolic conversion of methionine sulfone, either as free amino acid or in protein linkage, to methionine. However, the possibility remains that the methyl group of the sulfone may be available for transmethylation. A recent paper by Ványi-Nagy (6) gives evidence for an intermediary rôle of the sulfoxide in the demethylation of methionine. This author also implicates sulfone formation as an intermediary step in the process, although without experimental support.

An experiment mentioned by us earlier (3) showed pronounced growth of rats when a choline-free diet containing 15 per cent oxidized casein was supplemented with 0.36 per cent homocystine, suggesting the conclusion, at that time, that transmethylation may occur. It was subsequently found that under the conditions of this laboratory homocystine will induce growth without the benefit of methyl donors in the diet (7). In the light of this finding the conclusion that the methyl of methionine sulfone is available for methylation of homocystine became questionable.

Further study indicated that the ability of our rats to grow in the absence of known methyl donors is related to preexperimental nutritional conditions which evidently result in the storage of a factor or factors involved in methylation and possibly a synthesis of such factors by intestinal bacteria (8, 9). It became possible to abolish the homocystine-induced growth by modifying the intestinal flora of the animals by sulfonamide feeding and by depleting the rat of stored factors. Conditions were then established in which a dietary methyl donor, like choline, is required. The present investigation, conducted under such conditions, was undertaken to test the availability of the methyl group of methionine sulfone for methylation of homocystine. The sulfone was used as the free amino acid and in the protein linkage of oxidized casein.

Diet

The following compounds were used: DL-methionine, du Pont's product (99.9 per cent by perchloric titration), DL-methionine sulfone (10) (99.8 per cent by perchloric titration), oxidized casein (0.008 per cent methionine and 0.28 per cent cystine), and DL-homocystine (100.4 per cent by disulfide determination) prepared in this Laboratory by du Vigneaud's method modified by Brand (11, 12).

The composition of the basal amino acid diet was the same as that used in previous experiments and from the same sources (8). When oxidized casein was used instead of the 17 per cent amino acid mixture, it was added as 15 per cent of the basal diet and the adjustment was made in the percentage of dextrin. Sulfasuxidine (succinylsulfathiazole, Sharp and Dohme) was fed as 2 per cent of the diet, sucrose 15 per cent.

The vitamins were the same as those employed in previous experiments (8). Later in the experiment when the sulfonamide had become effective, 62.5 mg. of ryzamin-B unfortified (product *t*, Table I (8)), 2 γ of biotin, and finally 20 γ of crystalline folic acid (folvite, Lederle) were added per rat per day to the B vitamins fed.

EXPERIMENTAL

Methionine Sulfone—Four female rats were used, two pairs of litter mates born within 5 days of each other. They were 34 and 39 days old when placed on the experimental diet.

The preexperimental diet and experimental conditions were the same as those previously described (8).

The rats were allowed to lose weight for 14 days on the basal synthetic diet containing sulfasuxidine. This period was extended from 8 to 14 days in order to deplete the animals as thoroughly as possible. Then 0.83 per cent homocystine was added to the diet and the basal food was restricted to 3 gm. Growth ensued and continued for 9 to 11 days, when the usual drop in weight (7) occurred, an average loss of 13 gm. in 5 days. At this point doses of ryzamin-B (62.5 mg.) and folic acid (20 γ) were administered for 3 days in order to aid recovery. The rats were then kept on the original B vitamin supplement and given the basal food *ad libitum* until growth leveled off. It was assumed that the sulfasuxidine had become effective and therefore, at this point, ryzamin-B and additional biotin were added, followed in a short time by folic acid. If the growth curve of the rats remained level upon the addition of these extra vitamins, the animals were in a condition suitable for assay purposes. The four rats in this experiment were first used for several liver fraction assays. Rather than condition new animals, which would involve time and expense, it was decided to use these rats for the present experiment. The 0.83 per cent homocystine in the basal diet was replaced by 0.93 per cent methionine in order to test the condition of the animals by their ability to respond to methionine. Fig. 1 shows the average growth curve of these four rats and their daily basal food consumption. After 10 days on methionine (A) they were returned to homocystine; during the next 8 days the curve fell (B). For the following 15 days (C) 1.12 per cent methionine sulfone was added to the 0.83 per cent homocystine supplement in the basal diet. All rats failed to grow during this period; the average daily losses were 0.30, 0.40, 0.50, and 0.52 gm. per day. When the sulfone was replaced by 18 mg. of choline per day in the presence of 0.83 per cent homocystine (D), all animals responded with an immediate gain; the average was 1.4 gm. per day. The initial growth experiment with methionine sulfone (1) indicates that the sulfone is not toxic.

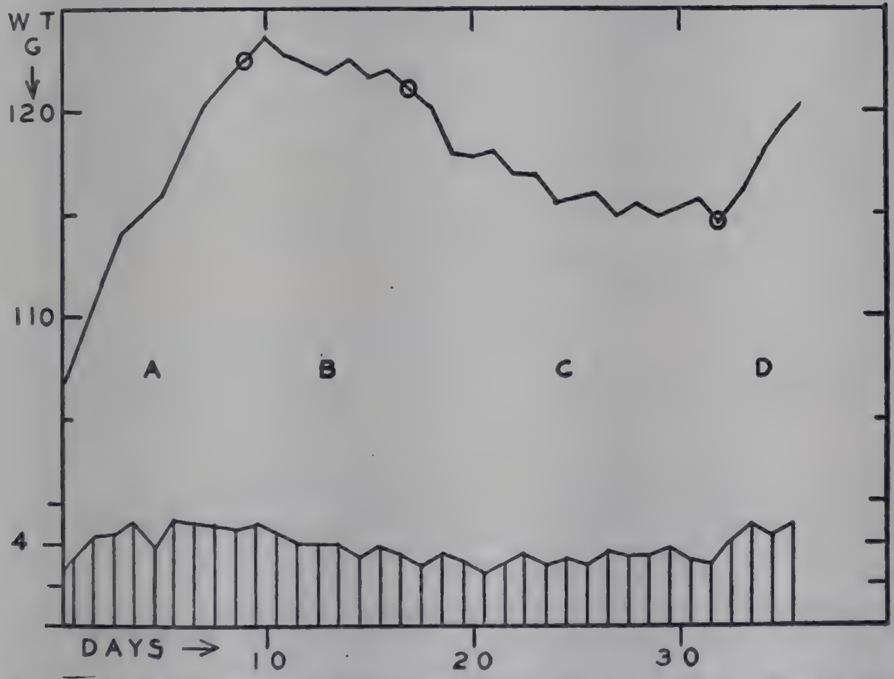

FIG. 1. Average daily growth and basal food consumption. Basal diet supplemented with the following: A, 0.93 per cent DL-methionine; B, 0.83 per cent homocystine; C, 0.83 per cent homocystine and 1.12 per cent methionine sulfone; D, 0.83 per cent homocystine and 18 mg. of choline chloride.

Oxidized Casein—Five female rats were used. One pair, litter mates, was 41 days old at the start of the experiment; the other three were from different litters, two of the three born the same day being 40 days old, the other, 37 days, when placed on the experimental diet.

These animals were given the same diet and depleted exactly as the four rats previously described. None of the five was used for liver fraction assay. When they showed a level growth curve on addition of the ryzamin-B, folic acid, and additional biotin, which required about 90 days, they were used for the present experiment. Fig. 2 shows the average growth curve and daily basal food consumption of these rats. The first period of 10 days (A) is the level period on the full vitamin supplement and 0.83 per cent homocystine. For the next 14 days (B) the 17 per cent amino acid mixture and 0.83 per cent homocystine diet was replaced by one containing 15 per cent oxidized casein supplemented with 0.4 per cent L-tryptophan and 0.83 per cent homocystine. There was an average gain of only 1.0 gm. during this period, the average daily changes of the five animals being −0.07, +0.04, +0.06, +0.14, and +0.16 gm. Upon returning to the amino acid diet containing 0.83 per cent homocystine, now supplemented with 18 mg. of choline chloride per day (C), gain resulted, the average being 1.9 gm. per day. A rat from a former experiment, which did not grow on a 15 per cent oxidized casein diet supplemented with 0.83 per cent homocystine and 0.4 per cent tryptophan, gained an average of 1.8 gm. per day for 9 days when 18 mg. of choline chloride were added per day to the oxidized casein diet.

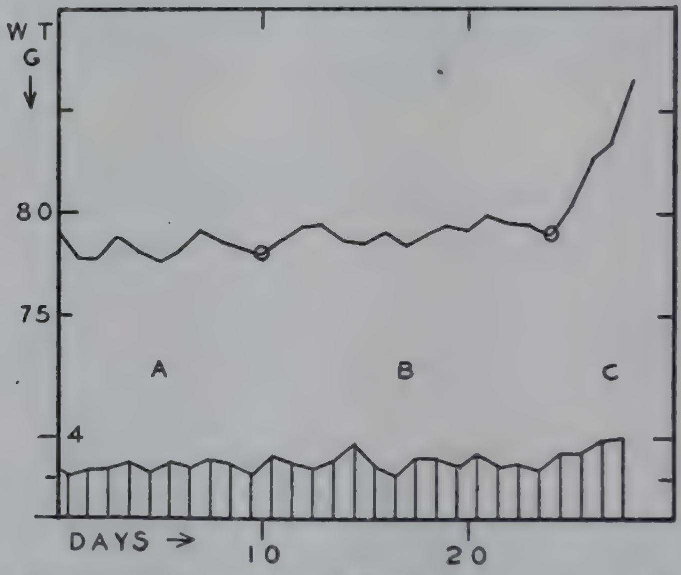

FIG. 2. Average daily growth and basal food consumption. A, basal amino acid diet with 0.83 per cent homocystine; B, 15 per cent oxidized casein, 0.4 per cent tryptophan, and 0.83 per cent homocystine; C, amino acid diet, 0.83 per cent homocystine and 18 mg. of choline chloride.

The fact that weight maintenance on the basal diet is somewhat better in the presence of oxidized casein than with the amino acid mixture may be due to the presence of strepogenin[1] or other protein factors.

SUMMARY

Rats fed a labile methyl-free homocystine diet supplemented by either methionine sulfone or oxidized casein failed to grow. This constitutes indirect proof of failure to methylate the homocystine and suggests that oxidation of methionine to the sulfone destroys the biological lability of its methyl group.

The author is indebted to Joseph J. Kolb for preparing the homocystine, methionine sulfone, and oxidized casein. She also wishes to thank Dr. G. H. Hitchings of the Wellcome Research Laboratories for the ryzamin-B unfortified, Dr. E. L. R. Stokstad of the Lederle Laboratories Division, American Cyanamid Company, for the folvite, and Dr. D. K. O'Leary of E. I. du Pont de Nemours and Company, Inc., for the DL-methionine.

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[1] A preparation of oxidized casein analyzed for strepogenin activity was found to be only slightly less active than casein (personal communication from D. W. Woolley to G. Toennies).

THE METABOLISM OF SOME BRANCHED CHAIN ALIPHATIC ACIDS

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Trimethylacetic (pivalic) acid and tertiary butylacetic acid, aliphatic acids in whose molecules a tertiary carbon atom is present, are not oxidized readily in the organism of the rat or rabbit, but appear to be excreted in considerable part in conjugation with glucuronic acid (1). These represent the first examples of the biological conjugation of glucuronic acid[1] with an *aliphatic acid.* We have been interested in further studies of aliphatic acids which are not oxidized readily in the animal body and which might conjugate similarly with glucuronic acid. These have included methylneopentylacetic acid, two α-substituted (ethyl group) aliphatic acids, and hydroxyisobutyric acid.

EXPERIMENTAL

The animals (rats, rabbits), the general laboratory procedures, and the methods used were the same as those of our previous studies from this laboratory (1). The acids were administered as the sodium salts either orally or subcutaneously.

Methylneopentylacetic Acid (2,4,4-Trimethylpentanoic Acid)[2]—This acid boiled at 216° and had a refractive index ($n_D^{20°}$) of 1.4228 and a density (at 25°) of 0.8882. These constants are in excellent agreement with those reported by Whitmore and associates (3).

Methylneopentylacetic acid (MNPA) is an α-methyl-substituted acid of the type which is usually stated to be oxidized readily in the animal organism (4, 5). Oxidation at the β-carbon atom, as postulated by Carter (p. 50 (5)), should yield trimethylacetic acid; demethylation with subsequent α oxidation should yield tertiary butylacetic acid. According to our earlier observations (1), if either of these acids were formed, they should be resistant to further metabolic change and should be excreted in conjugation with glucuronic acid. We have not been able as yet to isolate either of these acids or their conjugation products from the urine of animals fed MNPA. Our data indicate that a considerable portion of the acid, when administered to either rats or rabbits, is excreted without oxidation, and presumably in conjugation with glucuronic acid. As shown in Table I, when 1 gm. of the sodium salt of the acid was fed or injected subcutaneously to rabbits, an increased excretion of glucuronic acid, which corresponded to 62 to 72 per cent of the MNPA administered, was observed. Similar results were obtained with rats in which the increased glucuronic acid excretion was equivalent to from 22 to 48 per cent of the MNPA (100 mg.). The experimental urines reduced Benedict's solution, which sug-

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* Government of India Scholar.

[1] For a discussion of biological detoxication, in which conjugation with glucuronic acid is included, the excellent presentation of Williams (2) should be consulted.

[2] Obtained from the Mallinckrodt Chemical Works of St. Louis through the cooperation of Dr. Melvin J. Thorpe.

TABLE I

Excretion of Extra Glucuronic Acid after Administration of Methylneopentylacetic, α-Ethylbutyric, and α-Ethyl-n-caproic Acids

1 gm. of the sodium salt of each acid was administered orally except where subcutaneous injection is indicated. Glucuronic acid is calculated as glucurone. In the last two columns, the amount of acid conjugated is calculated on the assumption that all of the extra glucuronic acid is conjugated mole for mole with the aliphatic acid fed. Per cent refers to the percentage of the amount administered thus conjugated.

Acid	Experiment No.	Extra glucuronic acid	Aliphatic acid conjugated	
		mg.	mg.	per cent
Methylneopentylacetic (0.867 gm.)*	1-2	690	564	65
	2-1	759	621	72
	2-3†	655	536	62
	2-4	677	554	67
	2-5	755	618	70
	2-6	712	538	67
	2-7	739	605	70
α-Ethylbutyric (0.708 gm.)*	1-1	60	39	5
	2-2	367	242	29
	2-4†	312	206	25
	2-5†	658	433	52
	3-1	489	321	38
α-Ethylcaproic (0.867 gm.)*	4-1	880	719	83
	4-2	913	747	86
	4-3	896	733	85
	4-4	907	742	86
	4-5†	919	752	87

* Amount administered calculated as free acid.

† Subcutaneous administration.

gested the presence of the ester type of glucuronide, unstable to alkali, which splits off glucuronic acid and thus shows reducing properties with alkaline copper reagents.

No attempt was made to isolate the conjugated glucuronate from the urine. It was possible, however, by the use of the procedure previously described (1), to prepare an S-benzylthiuronium (SBT) salt from the experimental urines. The melting points and nitrogen contents of these salts are given in Table II. The data indicate that, like the other aliphatic acids with tertiary carbon atoms previously studied, MNPA escapes oxidation, in large part, in the animal body and is excreted in combination with glucuronic acid.

TABLE II

Melting Points and Nitrogen Contents of S-Benzylthiuronium Salts of Pure Acids and of Acids Isolated from Urines

The melting points are uncorrected. Mixed melting points of the derivatives of the pure acids and those isolated from the urines were satisfactory.

Acid	Sample No.	M.p.	Nitrogen
		°C.	*per cent*
Methylneopentylacetic			9.03*
	18†	155	8.98
	1	153–154	8.94
	4	154–155	8.96
	10	155	8.98
	13	151–153	9.01
α-Ethylbutyric			9.92*
	17†	142	9.73
	6	142	9.63
	7	142	9.68
α-Ethylcaproic			9.03*
	19†	132	8.96
	20	131	9.13

* Theoretical value.

† Derivative of pure acid.

α-Ethylbutyric Acid (2-Ethylbutyric Acid, Diethylacetic Acid)[2]—The acid used in the experiments boiled at 193.5° (uncorrected), which checked with the boiling point of 190–197° recorded in the literature. The data are presented in Tables I and II. The oral or parenteral administration of α-ethylbutyric acid to rabbits was followed by an excretion of considerable amounts of extra glucuronic acid (except in Experiment 1-1). The increases, while not as great as were noted when the acids containing a tertiary carbon atom were administered, correspond to from 25 to 52 per cent of the acid ingested (excluding Experiment 1-1). Similarly when 100 mg. of the sodium salt were fed or injected into young white rats, similar increases of glucuronic acid, corresponding to from 22 to 49 per cent, were observed. From the urines, it was possible to isolate a SBT salt which was identified as that of α-ethylbutyric acid by melting points and analyses for nitrogen (Table II).

In a single experiment in which the salt of α-ethylbutyric acid was injected subcutaneously into a dog, Blum and Koppel (6) have reported the excretion of methylpropyl ketone in the urine. It has also been stated that the acid gives rise to acetoacetic acid when perfused through the liver (4). Baer and Blum (7) believed that the urinary excretion of acetone bodies was increased after the feeding of α-ethylbutyric acid to human diabetics. In reviewing this work, Blum and Koppel (6) have pointed out that the urine of these patients also contained increased amounts of volatile substances which gave a positive iodoform test and that the urines were levorotatory. Since many of the glucuronic acid conjugates isolated from the urine are known to be levorotatory (Williams (2) pp. 122, 193), this suggests the presence of derivatives of glucuronic acid in these urines.

In view of the observations of Blum and Koppel, we have attempted to detect the presence of ketones, notably methylpropyl ketone, in the experimental urines by the reaction with 2,4-dinitrophenylhydrazine. We were unable to establish the presence of ketones in increased amounts even in Experiment 1-1, in which the excretion of extra glucuronic acid and the amounts of α-ethylbutyric acid isolated as the SBT salt were small.

If the fate of α-ethyl-substituted acids is similar to that postulated for those with α-methyl substituent groups (4, 8), butyric acid should be the product of metabolism, and should yield acetoacetic acid with liver slices and acetone in the diabetic organism. On the other hand, if β oxidation preceded dealkylation, the product, a β-ketonic acid ($CH_3—CO—CH(C_2H_5)—COOH$) would yield methylpropyl ketone by loss of carbon dioxide (4, 8). We have been unable to obtain evidence of either of these reactions in our experiments. Our data point to the excretion of a considerable portion of the α-ethylbutyric acid administered, in combination with glucuronic acid, although they do not exclude the possibility of the other two paths of catabolism.

α-Ethylcaproic Acid (*Butylethylacetic Acid, 2-Ethylhexoic Acid*)[3]—The acid distilled at 218-220° at 736 mm. pressure and was a colorless liquid, almost insoluble in water, which gave a neutral equivalent of 145.2 (theoretical, 144). For the preparation of the sodium salt, an alcoholic solution of the acid was neutralized (phenolphthalein indicator) with alcoholic sodium hydroxide; the solvent was removed under diminished pressure and the residue was dried at 90° for 48 hours. The hygroscopic material was used in the feeding experiments. The SBT salt, prepared in the usual manner, recrystallized from hot benzene and dried, melted at 132° (uncorrected) and contained 8.94 per cent of nitrogen (theoretical, 9.03 per cent).

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[3] Obtained through the courtesy of the Carbide and Carbon Chemicals Corporation.

When α-ethylcaproic acid was fed or injected subcutaneously into a rabbit, an increased excretion of glucuronic acid resulted, an increase which corresponded to 83 to 87 per cent of the acid administered (Table I). This excretion of glucuronic acid represented a higher conjugation of the organic acid than has been observed either in the present series of experiments or in those with pivalic and tertiary butylacetic acids previously reported from this laboratory (1). No significant excretion of acetone bodies was observed.

Our efforts to prepare the SBT salt of the acid from the urine were less successful than in our earlier experiments (1). From the ether-extractable material obtained from the acidified urine, a gummy product was obtained in the reaction with SBT chloride. This was extracted with hot benzene, in which the SBT salt of α-ethylcaproic acid is soluble readily, and on evaporation of the benzene, small amounts of a salt were obtained which appeared to be the SBT derivative of α-ethylcaproic acid (Table II). In another experiment, the ether extract of the acidified urine was evaporated to dryness and extracted with petroleum ether. From the petroleum ether fraction, an SBT salt melting at 131° was obtained. The petroleum ether fraction, however, represented only about one-twelfth of the material extractable from the urine by ether and titratable with alkali. We are unable to offer any satisfactory explanation of the low yield of the SBT salt despite the marked increase in the glucuronic acid content of the urine. Glucuronides of this type are readily broken down. It is possible that the conjugated glucuronide if formed with the fatty acids with longer carbon chains may be less labile and that the α-ethylcaproic acid is not free to react with the SBT chloride. This needs further study.

Hydroxyisobutyric Acid (*Acetonic Acid*)—The acid, prepared by saponification of the methyl ester[4] and recrystallized from boiling benzene or petroleum ether, melted at 79° (uncorrected) and had a neutral equivalent of 104.9 (theoretical, 104). From the acid, the sodium salt was prepared, recrystallized from boiling alcohol, and dried at 110° for use in the animal experiments. From the sodium salt, the SBT derivative was prepared in the usual manner (1) and after recrystallization from a mixture of benzene and acetone (4:1) and drying, melted at 141° (uncorrected), and contained 10.25 per cent of nitrogen (Kjeldahl) (theoretical, 10.37 per cent).

For the quantitative estimation of the acid, the reaction with ferric chloride served as the basis. The yellow color obtained resembles that given by lactic acid with the same reagent. To amounts of the acid ranging from 0.25 to 2.0 mg. in 10 ml. of water was added 1 ml. of a 1 per cent ferric chloride solution (freshly diluted from a 10 per cent stock solution). The color which developed immediately was read within 5 minutes in a Klett-Summerson photoelectric colorimeter (Filter 42). From these readings, a standard curve was prepared.

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[4] Obtained through the courtesy of the Rohm and Haas Company of Philadelphia.

For the determination of the acid in the urine, 5 to 10 ml. of urine were acidified with 2 ml. of 10 per cent phosphoric acid and extracted for 2 hours with ether in the extraction apparatus described by Griffith (9) for the extraction of hippuric acid from urine. 10 ml. of distilled water were added to the ether extract and the ether was removed by evaporation on a steam bath and finally under slightly diminished pressure. The aqueous solution remaining was made up to a definite volume and aliquots were used in the ferric chloride reaction as described. Normal urines similarly treated served as blank determinations. By these procedures, it was possible to recover from 91 to 96 per cent of hydroxyisobutyric acid added as the sodium salt to urine and from 95 to 98 per cent from aqueous solutions of the salt.

1 gm. of the sodium salt dissolved in water was fed through a stomach tube or injected subcutaneously into a male rabbit of approximately 2.4 kilos. This amount even when administered daily over a period of 4 days produced no obvious signs of toxicity; the animals ate the food offered regularly and completely. The urine was collected in 24 hour periods and analyzed as already described. In one experiment, the partition of urinary sulfur was determined by the usual procedures. The data are summarized in Table III.

The experimental urines did not reduce Benedict's copper solution, nor give a test for acetone bodies. There was no increase in glucuronic acid excretion nor in conjugated sulfate sulfur. The total nitrogen and creatinine excretions (not shown in Table III) were remarkably constant. The amount of ether-soluble material which reacts in the ferric chloride test was equivalent to 35 to 42 per cent of the acid administered. Since lactic acid also reacts with ferric chloride and would be determined by our procedure, an attempt was made to identify the substance in the urine which gave the yellow color with ferric chloride.

A rabbit was fed 1.5 gm. of the acid (in solution in the theoretical amount of sodium bicarbonate) daily for 2 days. The pooled urines were acidified with phosphoric acid and extracted with ether for 8 hours. The ether extract was dried with a small amount of anhydrous sodium sulfate, was shaken with decolorizing charcoal, and filtered. The residue, after evaporation of the ether, was converted to the sodium salt (1.15 gm. of crude dried material). 0.5 gm. of this salt was converted to the free acid which was crystallized from petroleum ether. Somewhat more than 250 mg. of the acid were obtained. The crystals melted at 79°, did not depress the melting point of a sample of the pure acid, and, when analyzed by the ferric chloride reaction, showed a purity of approximately 98 per cent.

From the remainder of the sodium salt prepared from the urine, the SBT salt was prepared. 800 mg. of a derivative, which melted at 140° and did not depress the melting point of a similar derivative of the pure acid, were thus prepared. On analysis (Kjeldahl), 10.45 per cent of nitrogen was obtained (theoretical, 10.37 per cent). It is evident from these observations that the major part of the material excreted in the urine and reacting with ferric chloride was the unchanged acid. The possibility of the presence of small amounts of lactic acid cannot, however, be ruled out entirely.[5]

Table III

Glucuronic and Hydroxybutyric Acid Contents of Urine after Administration of Hydroxybutyric Acid

1 gm. of the sodium salt (equivalent to 0.825 gm. of the acid) was administered orally except as indicated. Glucuronic acid is calculated as glucurone.

Period	Glucuronic acid	Hydroxyisobutyric acid	
	mg.	*mg.*	*per cent of intake*
Control*	131 (101–178)		
Experimental	153	290	35
" †	101	337	41
"	114	350	42
" ‡	152	341	41

* The average daily excretion of 11 control days. The values in parentheses represent the range of normal values.

† The partition of sulfur showed excretions of 69 mg. of inorganic sulfate and 7 mg. of conjugated sulfate sulfur on the experimental day, in comparison with similar values of 79 and 7 mg., respectively, on 3 control days immediately preceding and following the experimental day.

‡ Subcutaneous administration.

Previous studies of the fate of hydroxyisobutyric acid in the animal organism are limited and the data are not presented in detail. Blum in 1910 (11) reported that when the acid was administered to young animals

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[5] The SBT salt of lactic acid melts at 153° (10) and has a nitrogen content of 10.93 per cent. While the differences between the melting point and nitrogen content of the SBT salts of lactic acid and hydroxyisobutyric acid are not great, it is believed that the properties of the SBT salt of the acid isolated from urine exclude the presence of a significant amount of the lactic acid salt. When the SBT salts of lactic acid prepared in this laboratory and of the ether-soluble acid, extracted from the experimental urines, were mixed, the melting point of the mixture was 127–135°, further evidence of the absence of lactic acid in the SBT salt prepared from urine.

(neither the route of administration nor species of animal is stated); acetoacetic acid was formed, but the acid was excreted, largely unchan together with traces of lactic acid. Since approximately 60 per cent of acid fed in our experiments could not be accounted for in the urine, regar less of the mode of administration, we believe that this fraction has pr ably undergone biological oxidation. In such an oxidation, the first s might well be demethylation with the formation of lactic acid, which sho not appear in the urine. It is of interest to note that hydroxyisobuty acid is reported not to be metabolized by microorganisms (12).

The behavior of hydroxyisobutyric acid is in striking contrast to t of isobutyric and pivalic (trimethylacetic) acids. Isobutyric acid gave to extra glucose in amounts approximately equivalent to 3 carbon atom the phlorhizinized dog (13). Pivalic acid was excreted, largely unchang but in combination with glucuronic acid by the normal rabbit (1). I also of interest to note that β-hydroxyisovaleric acid, which contains grouping $(CH_3)_2C(OH)$— present in hydroxyisobutyric acid was oxidi in living tissue and is reported to give rise to acetoacetic acid in perfus experiments with the surviving liver (14).

SUMMARY

Methylneopentylacetic acid, an acid which contains a tertiary car atom, like the similar acids, trimethylacetic and tertiary butylacetic, oxidized with difficulty in the organism of the rabbit and the rat and administration led to the excretion of large amounts of extra glucur acid, presumably in conjugation with the unchanged acid.

The administration of two acids in whose molecule an ethyl group substituted on the α-carbon atom, α-ethylbutyric and α-ethyl-n-cap acids, also resulted in the excretion of considerable amounts of extra g curonic acid. This suggests the presence of the unchanged acid a glucuronide and is in accord with a few early observations which indica that such acids were oxidized with difficulty (in contrast to α-met acids).

No ketone derivatives were isolated when hydroxyisobutyric acid administered; considerable amounts of the acid were excreted as such, no conjugation with glucuronic acid was indicated.

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THE OXIDATION OF UNSATURATED FATTY ACID IN NORMAL AND SCORBUTIC GUINEA PIGS

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Kohn and Liversedge (1) showed that rat tissues incubated aerobically formed a substance which reacts with either *p*-aminobenzoic acid (PAB) to give a yellow color or with thiobarbituric acid (TBA) to give an orange-red color. Bernheim, Wilbur, and Fitzgerald (2) found that ascorbic acid catalyzes the oxidation of a compound which is linked to brain protein, and that the oxidation product reacts with these reagents producing the characteristic colors. Ascorbic acid is known to catalyze the oxidation of lipides. Rusch and Kline (3) demonstrated this for liver phospholipides; Elliott and Libet (4) found that phospholipides increased the oxygen consumption of brain suspensions and that this effect is augmented by ascorbic acid. That the substance which is linked to tissue protein and reacts with PAB and TBA is lipide is indicated by the recent work of Bernheim, Bernheim, and Wilbur (5), in which it was found that the oxidation products of certain unsaturated fatty acids form the colored compounds. Analysis of the TBA compound showed that a 3-carbon fragment had combined with the thiobarbituric acid. The reaction is given by lecithin, cephalin, and methyl linolenate but not by lysolecithin, oleic, or linoleic acid. The absorption spectrum of the colored compound formed by methyl linolenate with thiobarbituric acid is identical with that given by tissues,[1] and it is presumed that the substance concerned is linolenic or a related fatty acid from which a 3-carbon fragment can be split.

The thiobarbituric acid test provides a convenient method for following the oxidation of linolenic and possibly similar fatty acids and has been utilized in the present experiments to study the influence of ascorbic acid on fatty acid oxidation in several tissues of normal and scorbutic guinea pigs. In most scorbutic tissues a subnormal oxidation has been found. Addition of ascorbic acid to such tissues *in vitro* increased the amount of oxidation to the normal level. This indicates that ascorbic acid is a catalyst for this oxidation and that the tissues of the scorbutic animal contain normal amounts of fatty acid.

EXPERIMENTAL

Male guinea pigs weighing 260 to 480 gm. were given a scorbutigenic diet (6) mixed with minced, fresh carrots plus a daily oral supplement of 5 mg. of crystalline ascorbic acid (6). After 2 to 8 days the animals had become accustomed to the new diet and were then divided into experimental and control groups on the basis of similar weights. The experimental group, which was given a diet lacking ascorbic acid, showed typical symptoms of scurvy after 2 to 3 weeks (7). At that time both the control and experimental groups were given 0.5 gm. of L-tyrosine orally, and the urines were tested for the presence of hydroxy acids (6). After the scorbutic animals gave a positive test, the normal and deficient guinea pigs were decapitated. Postmortem examination revealed no symptoms of scurvy in the control animals (7).

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[1] Bernheim, F., and Wilbur, K. M., personal communication. Tested on fresh sample supplied by the Nutritional Biochemicals Corporation.

Organs to be studied were dissected immediately, weighed, ground thoroughly in a mortar, squeezed through muslin, and diluted with 0.05 M Na-K phosphate buffer of pH 6.7 to give a concentration of 200 mg. of tissue per ml. of buffer. To 1.5 ml. of buffer was added 0.5 ml. of tissue suspension, and the flasks were incubated at 37–38° for 105 minutes with shaking. For testing the effect of ascorbic acid, 0.5 mg. was added to the suspension (2). The pH remained at 6.7. After incubation 1.0 ml. of 20 per cent trichloroacetic acid and 4 ml. of thiobarbituric acid reagent (1) were added to the suspension, which was then placed in a boiling water bath for 5 minutes. The precipitate was centrifuged, and the density of color of the decanted liquid was read in a Fisher electrophotometer with Filter 525B. Blanks were run to correct for the reagents and the turbidity of the tissue suspensions.

Results

Fatty acid oxidation of tissues of normal guinea pigs on a controlled diet, as measured by the amount of color produced, is summarized in Table I, Column 4. Brain gave the highest values followed in order by testis, medulla oblongata, adrenal, kidney, liver, and spleen, with very low values for heart. In normal animals on a normal uncontrolled diet,[2] the sciatic nerve gave values comparable with brain and testis. Oxidation was similar in various parts of the brain except for the olfactory bulbs in which it was much reduced, a finding which may be related to the smaller number of myelinated fibers. Values for adipose tissue were in the same range as adrenal and kidney. Throughout the experiments care was taken to remove all extraneous fat from the organs before the tissue suspensions were made.

Fatty acid oxidation in scorbutic whole brain, medulla oblongata, testis, adrenal, and kidney was significantly less than that in the corresponding normal tissues (Columns 2 and 4).[3] Addition of ascorbic acid to these tissues of the scorbutic animals brought the values into the normal range (Columns 2, 3, and 4). On the other hand, scorbutic liver, spleen, and heart gave essentially normal values, and the addition of ascorbic acid to these tissues caused no significant change except some decrease in scorbutic spleen. Several normal tissues exhibited increases on the addition of

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[2] The normal uncontrolled diet consisted of Rockland guinea pig diet, hay, and fresh carrots and lettuce.

TABLE I

Thiobarbituric Acid Color Production by Normal and Scorbutic Guinea Pig Tissues Incubated with and without Added Ascorbic Acid

The tissue suspensions were incubated 105 minutes at 37–38°. The color density was measured with a Fisher electrophotometer with Filter 525B. Scale readings above 5 were directly proportional to concentration within the range employed. The figures represent mean values with probable error. All tissues listed below "heart" are from normal animals on a normal uncontrolled diet.

Tissue	No. of normal or scorbutic guinea pigs (1)	Scorbutic (2)	Scorbutic with ascorbic acid added (3)	Normal (4)	Normal with ascorbic acid added (5)
Brain	9	29.9 ± 1.2	49.2 ± 2.3	42.4 ± 0.9	50.9 ± 4.0
Medulla oblongata	3	10.6 ± 1.0	37.6 ± 5.0	24.0 ± 1.9	34.6 ± 4.6
Testis	9	17.4 ± 1.5	41.5 ± 3.3	34.1 ± 1.3	41.2 ± 4.3
Adrenal	6	7.9 ± 0.7	19.2 ± 5.2	20.9 ± 2.8	14.4 ± 4.1
Kidney	9	8.1 ± 0.9	39.5 ± 3.2	16.9 ± 0.7	47.0 ± 2.4
Liver	9	15.3 ± 3.6	19.8 ± 6.4	10.7 ± 2.2	4.3 ± 0.6
Spleen	9	8.8 ± 0.6	6.2 ± 0.6	10.2 ± 0.6	8.4 ± 1.0
Heart	3	1.2 ± 0.2	2.1 ± 0.5	1.9 ± 0.3	0.5 ± 0.3
Right sciatic nerve	3			48.1 ± 6.5	53.7 ± 6.2
Left " "	3			29.7 ± 5.7	43.2 ± 7.6
Right olfactory bulb	4			3.9 ± 1.0	
Left " "	3			2.2 ± 0.7	
Right cerebral hemisphere	4			16.5 ± 1.7	
Left cerebral hemisphere	4			16.6 ± 1.9	
Cerebellum	4			11.2 ± 1.4	
Mesencephalon and diencephalon	4			15.5 ± 0.4	
Pituitary	4			16.9 ± 7.5	
Medulla oblongata	3			12.9 ± 2.0	
Fat posterior to right kidney	3			19.7 ± 3.3	24.2 ± 4.7

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[3] The significance of the difference between the means in Table I has been tested. This includes a comparison of the following sets of data: scorbutic-normal, scorbutic with ascorbic acid added-normal, scorbutic-scorbutic with ascorbic acid added, and normal-normal with ascorbic acid added.

ascorbic acid (Columns 4 and 5). The differences are statistically significant for kidney and doubtful for brain. Liver showed a decrease of doubtful statistical significance.

DISCUSSION

The oxidation of fatty acid and lipides *in vitro* proceeds spontaneously in the presence of oxygen and is catalyzed by ascorbic acid and hemoglobin (3–5, 8), and in the organism it is not unlikely that these same factors may have an influence on the oxidation of fatty acid, whether free, part of the phospholipide molecule, or combined with protein. That ascorbic acid plays an important part in the oxidation of linolenic acid in tissues is indicated by the present study in which certain scorbutic tissues exhibited a deficient oxidation *in vitro* which could be corrected by the addition of ascorbic acid. The deficiency may well be greater than the results suggest, since hemoglobin present in the tissue suspensions would also catalyze the oxidation. Accordingly, it appears probable that the oxidation of linolenic, and possibly other closely related acids, is considerably retarded in certain tissues of the scorbutic animal.

The results also suggest a possible method for measuring the ascorbic acid saturation of individual tissues with respect to linolenic acid oxidation. If addition of ascorbic acid to a tissue does not increase the amount of oxidation, the tissue may be said to be saturated. If there is an increase, then the amount of ascorbic acid required to give a maximal value might be used as a measure of the deficiency. On this criterion certain tissues of guinea pigs receiving 5 mg. of ascorbic acid per day are not saturated, while liver, spleen, and heart, on the other hand, may show maximal values even in scorbutic animals (Table I).

The thiobarbituric acid test may provide a means of measuring the linolenic acid content of tissues. The reagent gives a measurable color with as little as 1 γ of pure methyl linolenate.[1] On the basis of the color reaction, 100 mg. of normal guinea pig brain, a tissue with a high content, would contain only about 50 γ. It is possible that the failure to find linolenic acid in various animal tissues may be due to the insensitivity of the previous methods used.

For certain of the results listed in Table I, no satisfactory explanation is apparent. This applies to the inhibitory action of added ascorbic acid on normal liver and scorbutic spleen. Also, values for various parts of the brain were much lower than expected on the basis of analyses of whole brain.

The author appreciates the helpful suggestions of Dr. Frederick Bernheim and Dr. Karl Wilbur and the aid of Dr. W. L. Deemer, Jr., with statistical procedures.

SUMMARY

1. The oxidation of unsaturated fatty acid was measured by the thiobarbituric acid color reaction in normal and scorbutic guinea pig tissues which were incubated with and without added ascorbic acid. In tissues from normal guinea pigs oxidation was greatest in brain, nerve, and testis, followed by medulla oblongata, adrenal, kidney, fat, liver, and spleen, with very low values for heart and olfactory bulbs.

2. In tissues from scorbutic guinea pigs oxidation was decreased in brain, testis, medulla oblongata, adrenal, and kidney, whereas oxidation in liver, spleen, and heart was not significantly different from normal. The subnormal oxidation of scorbutic tissues was corrected by the addition of ascorbic acid *in vitro*.

3. The addition of ascorbic acid to tissues of normal animals increased oxidation in some, gave no change in others, and caused a decrease in liver.

4. A method for estimating the ascorbic acid saturation for fatty acid oxidation in individual tissues is suggested and consists of a determination of the amount of ascorbic acid required to give maximal oxidation on incubation, as indicated by the thiobarbituric acid color reaction.

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MAMMALIAN TYROSINASE: PREPARATION AND PROPERTIES*

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The enzyme tyrosinase, which catalyzes the aerobic oxidation of tyrosine to produce the pigment melanin, is found widely distributed in nature. Much has been reported (1, 2) on tyrosinase obtained from plant, insect, and marine animal sources, but relatively little is known concerning mammalian tyrosinase. Some years ago, Bloch and his coworkers proposed, on the basis of histochemical evidence, that melanin formation in mammalian skin was attributable to the presence of a specific enzyme whose substrate was not tyrosine but dihydroxyphenyl-L-alanine (dopa) (3). This enzyme was given the name "dopa oxidase." Largely as the result of this work, which has been amply confirmed, the concept has arisen that the primary amino acid precursor of mammalian melanin is not tyrosine but dopa, and there has even been some question as to the actual existence of a *mammalian* tyrosinase, although it is generally agreed that the enzyme from other sources is a true tyrosinase.

In 1903 Gessard (4) found that extracts from a horse melanoma were able to catalyze the conversion of tyrosine to melanin. This was confirmed by de Coulon in 1920 (5). In 1907 Alsberg (6) prepared an extract from a human melanoma which could catalyze the formation of black pigment from catechol and possibly from tyrosine. In the following year Neuberg (7) showed that dilute extracts from a human melanoma accelerated pigment formation from tyramine and adrenalin but not from tyrosine. Recently Hogeboom and Adams (8) demonstrated that extracts from the Harding-Passey mouse melanoma possess both tyrosinase and dopa oxidase activity. They reported the separation of these two activities by ammonium sulfate precipitation. Similar results were reported by Greenstein and Algire (9). In addition, Greenstein *et al.* (10) found that extracts from a human melanoma show both tyrosinase and dopa oxidase activity.

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The work reported here indicates that the enzymatic activity concerned in the oxidation of both tyrosine and dopa to melanin by extracts from the Harding-Passey mouse melanoma is associated with cell particles and is, therefore, not susceptible to separation by the common methods for fractionating mixtures of proteins. It is also shown that under certain conditions no true distinction can be made between tyrosinase and dopa oxidase activity in the mammalian tissue preparations used, thus militating against the possibility that these two activities can be ascribed to separate enzymes. In addition, it is shown that dopa is an intermediate in the oxidation of tyrosine by mammalian tyrosinase.

EXPERIMENTAL

Extracts from the Harding-Passey mouse melanoma, prepared as described below, were subjected to various fractionation procedures at 5° unless otherwise specified. Tyrosinase and dopa oxidase activities of the fractions were determined manometrically by measurement of the oxygen uptake in the Warburg apparatus at 38° with 0.1 M potassium phosphate buffer at pH 6.8. Autoxidation of dopa is negligible at this pH for the time intervals of the experiments. The substrates L-tyrosine (Eimer and Amend) and dihydroxyphenyl-L-alanine (Hoffmann-La Roche) were added from the side arms of the Warburg vessels to the enzyme preparations after 10 minutes equilibration at 38°. Enzymatic activity is expressed in units as proposed by Hogeboom and Adams (8); *viz.*, 1 activity unit is the amount of enzyme required to catalyze the absorption of 1 microliter of oxygen per minute by 1 mg. of appropriate substrate when oxidation is proceeding at a maximal rate. In general 2.5 to 4.5 units of enzyme were used per Warburg vessel containing 3 ml. of reaction mixture.

The definition of an enzyme unit as given above requires some explanation in the case of tyrosinase activity. It is well known that in the oxidation of tyrosine by tyrosinase there is usually a variable time interval (the "induction period") before oxygen uptake reaches a maximal rate. Calculations of tyrosinase activity in units are, therefore, based upon rates during the time of active oxidation rather than over-all time.

Ammonium Sulfate Fractionation—Fractionation with ammonium sulfate was attempted as described by Hogeboom and Adams (8). In a typical experiment, 18.5 gm. of melanoma tissue were ground with sand and Ringer's solution for 10 minutes, followed by centrifugation at 1500 R.P.M. for 5 minutes to remove sand and cellular débris. The supernatant fluid contained 790 units of tyrosinase and 790 units of dopa oxidase activity. This was then centrifuged at 20,000 R.P.M. for 10 minutes, and the precipitate discarded. The supernatant fluid at this point contained respectively 210 and 230 units of tyrosinase and dopa oxidase activity. On treatment with ammonium sulfate solution to one-third saturation, the precipitate so obtained contained 70 units of tyrosinase and 95 units of dopa oxidase activity. The supernatant fluid was then brought to two-thirds saturation with ammonium sulfate. The resulting precipitate contained no apparent tyrosinase activity, 110 units of dopa oxidase activity, and the supernatant fluid was inactive.

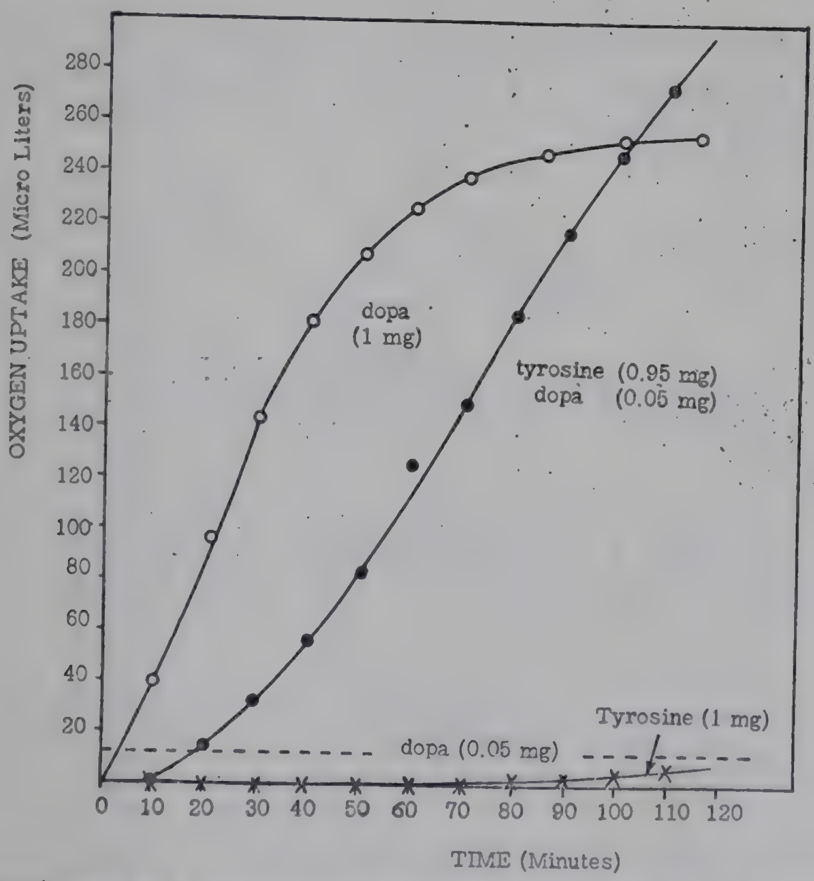


FIG. 1. Tyrosinase and dopa oxidase activity of tumor fraction not precipitated by one-third saturated ammonium sulfate, but precipitated by two-thirds saturated ammonium sulfate. pH 6.8 and 38°.

Hogeboom and Adams stated that their crude tumor extract possessed much greater dopa oxidase activity than tyrosinase activity, and that they were able to separate tyrosinase from dopa oxidase by precipitation at one-third and two-thirds saturation respectively with ammonium sulfate. In our experiments equal tyrosinase and dopa oxidase activities were found in both the crude tumor extract and in the fraction precipitated by one-third saturation with ammonium sulfate, and this latter fraction contained as much dopa oxidase activity as was found in the precipitate from two-thirds saturation with ammonium sulfate. In preliminary experiments it appeared that the fraction insoluble in two-thirds saturated ammonium sulfate solution did not have any tyrosinase activity. However, when small amounts of dopa were added to the tyrosine-enzyme reaction mixture, oxidation of tyrosine followed, as shown in Fig. 1. This finding is discussed in greater detail below.

Ethanol Fractionation—Since the ammonium sulfate fractionation did not yield a preparation possessing only tyrosinase or dopa oxidase activity, ethanol precipitation at −5° was tried (11). The alcohol concentration was varied from 0 to 40 per cent and the pH from 4.7 to 8.0. No fractiona tion could be obtained, since enzymatic activity was present in all th precipitates.

Table I

Fractionation of Melanoma Extract by Differential Centrifugation

40.5 gm. of melanoma ground with sand and alkaline saline, centrifuged at 1500 R.P.M. for 5 minutes and again for 10 minutes; the residue discarded in each case, to obtain supernatant solution S_0.

Fraction	Enzyme content		Induction period	Nitrogen content
	units per mg. dry weight	*total units*	*min.*	*per cent*
Solution S_0	1.0	4350	130	8.4
Solution S_0 centrifuged at 2500 R.P.M. for 20 min.				
Solution S_1	1.1	2960	156	9.2
Ppt. P_1	0.8	670	106	9.1
Solution S_1 centrifuged at 20,000 R.P.M. for 10 min.				
Solution S_2	1.3	1960	160	9.1
Ppt. P_2	0.7	810	102	9.9
Solution S_2 centrifuged at 20,000 R.P.M. for 90 min.				
Solution S_3	1.0	690	190	10.0
Ppt. P_3	1.2	490	115	8.2
Solution S_3 centrifuged at 20,000 R.P.M. for 150 min.				
Solution S_4	0.6	200	300	12.5
Ppt. P_4	1.2	140	200	10.5

Differential High Speed Centrifugation—This was carried out on th crude tumor mince, in general by use of alkaline saline solution as d scribed by Claude (12). Similar results were obtained with distill water. The results of a typical centrifugation procedure are given Table I, where enzymatic activity is expressed in terms of units as pr viously defined with 1 mg. of dopa as the substrate. The inducti period is defined as the intercept on the time axis of an extension of t slope of the oxidation rate curve when oxidation is proceeding maximally, and was determined with 1 mg. of tyrosine as the substrate and 3.2 enzyme units of each of the various fractions. All the fractions were dialyzed twice against 100 times their volume of cold distilled water for 48 hours before use.

It can be seen from Table I that, after centrifugation of the tumor extract for 20 minutes at 2500 R.P.M. and then for 10 minutes at 20,000 R.P.M., the supernatant fractions contained more total activity and more activity per mg. of dry weight than did the precipitates. However, after further centrifugation, the precipitates were more active on a dry weight basis. The supernatant fluid remaining after 150 minutes centrifugation at 20,000 R.P.M. possessed less than 5 per cent of the original total activity of the crude preparation; after 4 hours at this speed, the supernatant fluid was entirely inactive.

After centrifugation of the various fractions, the precipitates usually showed a shorter induction period in the oxidation of tyrosine than did the supernatant fractions, even when the latter contained more activity. There appears to be a factor which concentrates in the precipitate fractions and which shortens the induction period. This factor does not appear to be an enzyme, because it is stable to heat at 100° for 10 minutes at pH 6.8, and it is not "free dopa" because it is not removed by dialysis. Further work on the nature of this factor is in progress.

The nitrogen content of most of our fractions is about 10 per cent. This is similar to that found by Claude (12) for the cytoplasmic particulate material which he called microsomes.

It is evident from the above results that, although the induction period in tyrosine oxidation could be changed by various fractionation procedures, none of the methods could be used to concentrate enzymatic activity. Therefore, it was decided to use an easily prepared working standard for additional studies on the mechanism of enzymatic action. Such preparations were made as follows: Fresh mouse melanoma tissue was ground with sand and cold distilled water for 10 minutes. The mixture was placed in a Waring blendor at 5° for 10 minutes and then centrifuged at 3000 R.P.M. for 30 minutes. The black supernatant fluid was dialyzed twice against 100 times its volume of cold distilled water for 48 hours. The final solution had approximately 4 times the volume of the original tumor tissue, and corresponds somewhat to Solution S_0 in Table I. Preparations of this type were used in the studies which follow.

Stability of Enzyme—The enzyme preparations are stable in solution at 5° for 2 months with no apparent loss of activity. A slight loss of activity occurs after 6 hours at 38°. Heating the preparations for 10 minutes at 70° results in complete inactivation.

Lyophilization does not alter enzymatic activity. Such dried preparations remain active at room temperature for 1 month, and, when kept cold, for as long as, and possibly longer than, 10 months.

Dialysis against water at 5° has no effect on the enzyme.

Preparations may be kept in the cold in solutions ranging in pH from 4.7 to 8.0 for 24 hours, without loss of enzymatic activity when the reaction is carried out at pH 6.8. The addition of 0.1 M acetate buffer at pH 4.7 to the enzyme preparation produces a precipitate containing all of the active material.

Plant tyrosinase is inactivated during reactions with various hydroxyphenyl compounds (1). The mammalian tyrosinase studied here does not appear to be similarly inactivated. If dopa is added to a reaction mixture which has previously oxidized dopa to melanin, the rate of oxidation is the same as that of the original reaction.

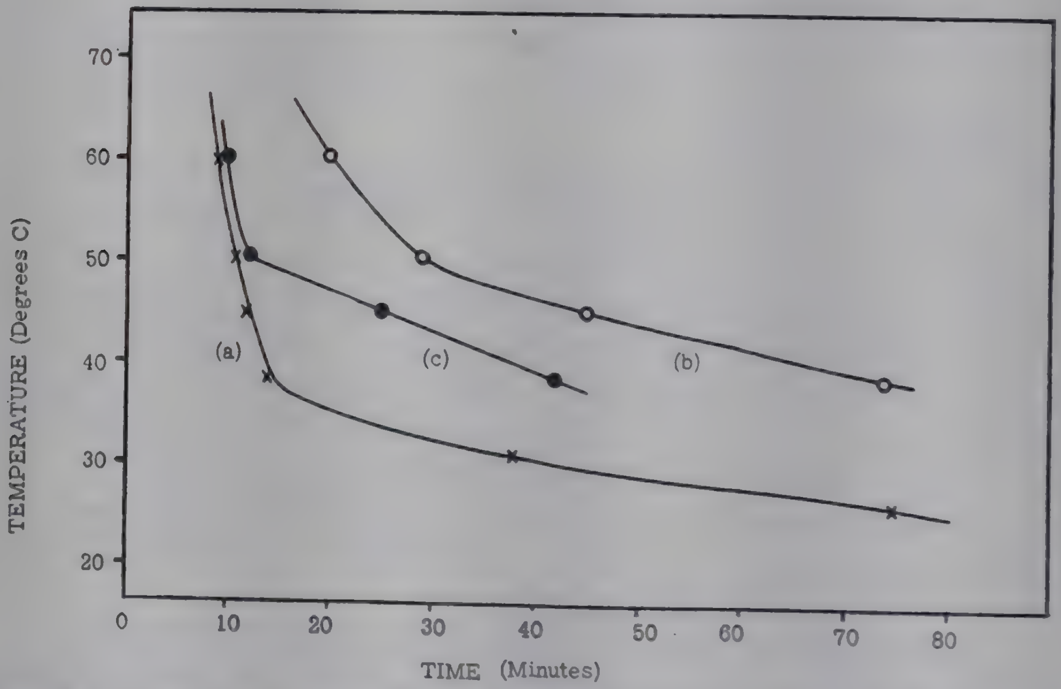


Fig. 2. Influence of temperature on the enzymatic oxidation of tyrosine and dopa by mouse melanoma extracts at pH 6.8. Curve *a*, time for 50 per cent oxidation of dopa; Curve *b*, time for 50 per cent oxidation of tyrosine, including the induction period; Curve *c*, induction period alone in the oxidation of tyrosine.

Effect of Temperature on Reaction Rates—In general, the rate of enzymatic oxidation of tyrosine and dopa increases with an increase in temperature (Fig. 2). An increase in temperature also shortens the induction period in the oxidation of tyrosine. The temperature coefficient for the enzymatic oxidation of dopa for the interval 27–37° is 1.2; for 37–47°, 1.7; and for 47–57°, 1.8. This variation in the temperature coefficient indicates that the oxidation of dopa to melanin is not a simple reaction, as indeed Evans and Raper have amply demonstrated for plant tyrosinase (13).

Effect of pH on Reaction Rates—The optimum pH for the enzymatic oxidation of dopa is at about pH 6.8. It is difficult to evaluate the effect of pH on the enzymatic oxidation of dopa above pH 7.0 because dopa is readily oxidized under these conditions, even in the absence of enzyme. At pH 5.0, a marked decrease in the rate of oxidation occurs.

The induction period in the enzymatic oxidation of tyrosine appears to be at a minimum at pH 6.8. At values above and below pH 6.8, the induction period increases, becoming prolonged indefinitely above pH 8.5 and below pH 5.0.

Effect of Substrate Concentration on Total Oxygen Uptake—The total oxygen uptake during the reaction is directly related to the initial amount of tyrosine (or dopa) present. If the amount of substrate is increased 2-fold, the total oxygen uptake is likewise increased 2-fold. The total amount of oxygen required to oxidize tyrosine and dopa to melanin is difficult to determine with great precision. Our results indicate that each tyrosine and dopa molecule requires approximately 5 and 4 atoms of oxygen respectively for conversion to melanin.

Induction Period in Tyrosine Oxidation—The concentration of tyrosine in the reaction mixture affects the induction period; the higher the tyrosine concentration, the longer the induction period.

Dopa has an opposite and a more dramatic effect on the induction period. The higher the dopa concentration, the shorter the induction period. This was demonstrated by adding varying amounts of dopa to reaction mixtures of tyrosine and enzyme and observing the course of oxygen uptake. The results are illustrated in Fig. 3. If the induction period is defined as mentioned above, one finds a linear relationship between the negative logarithm of the dopa concentration and the induction period (Fig. 4).

Identification of Dopa As an Intermediate in Tyrosine Oxidation by Mammalian Tyrosinase—Evans and Raper (13) have shown that when tyrosine is oxidized by plant tyrosinase dopa is an intermediate in the reaction. With the use of filter paper chromatography, we were able to show that dopa is an intermediate in the mammalian tyrosinase reaction also. The procedure of Consden, Gordon, and Martin (14) with phenol-water mixtures was used.

The solutions to be chromatographed were prepared by adding 1 mg. of tyrosine to a dialyzed enzyme preparation having an induction period of approximately 35 minutes. The reactions were carried out in ordinary Warburg vessels at 38° and pH 6.8. Small amounts of the reaction mixtures were removed at 0, 15, 30, and 50 minutes for chromatographic analyses. At 50 minutes, the oxygen uptake was at a rapid and linear rate, and about 50 per cent of the total oxygen absorbed in a completed

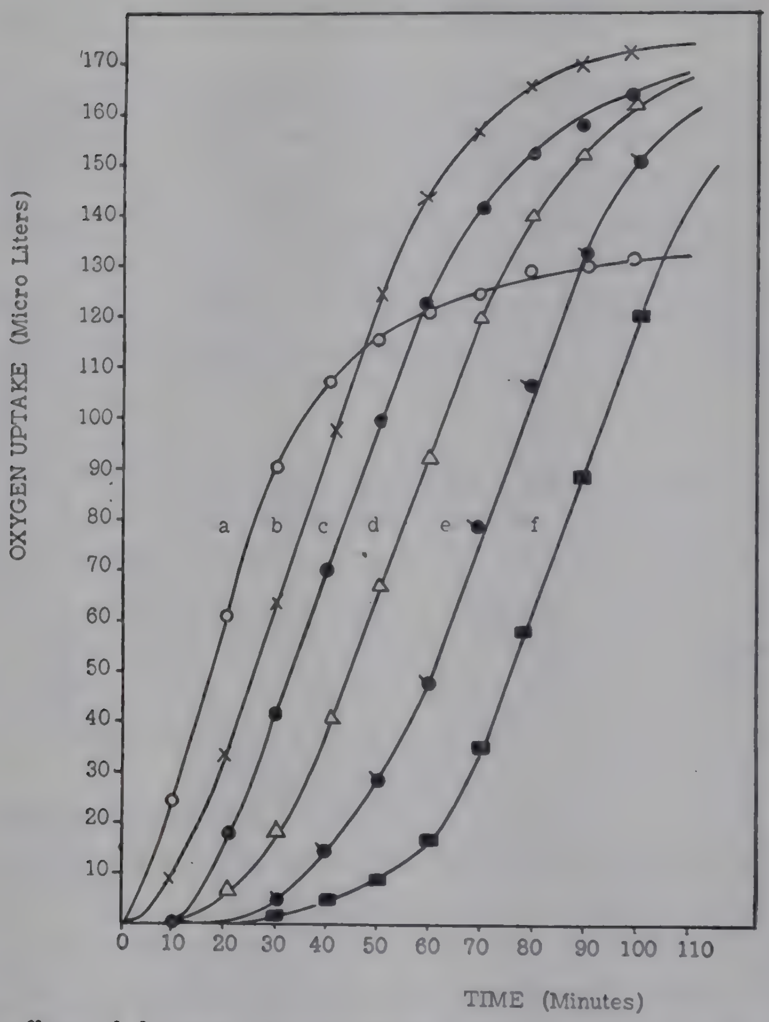


FIG. 3. The effect of dopa on the induction period in the enzymatic oxidation of tyrosine by mouse melanoma preparations at pH 6.8 and 38°. Curve *a*, 0.5 mg. of dopa; Curve *b*, 0.1 mg. of dopa + 0.4 mg. of tyrosine; Curve *c*, 0.05 mg. of dopa + 0.45 mg. of tyrosine; Curve *d*, 0.01 mg. of dopa + 0.49 mg. of tyrosine; Curve *e*,0.001 mg. of dopa + 0.50 mg. of tyrosine; Curve *f*, 0.50 mg. of tyrosine.

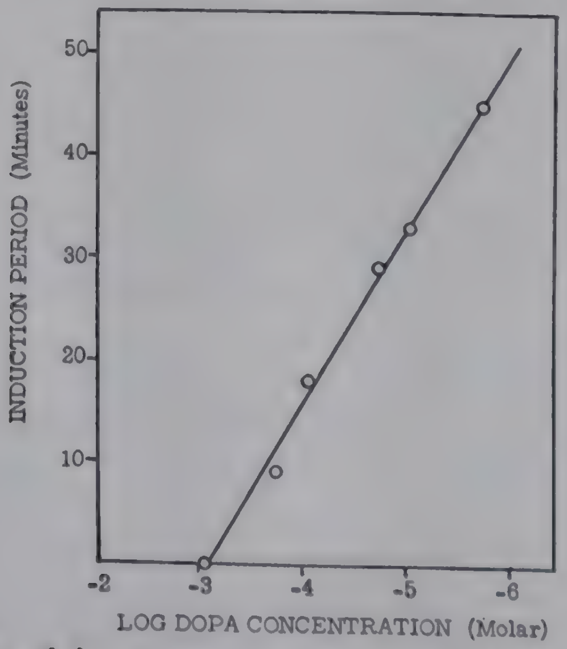


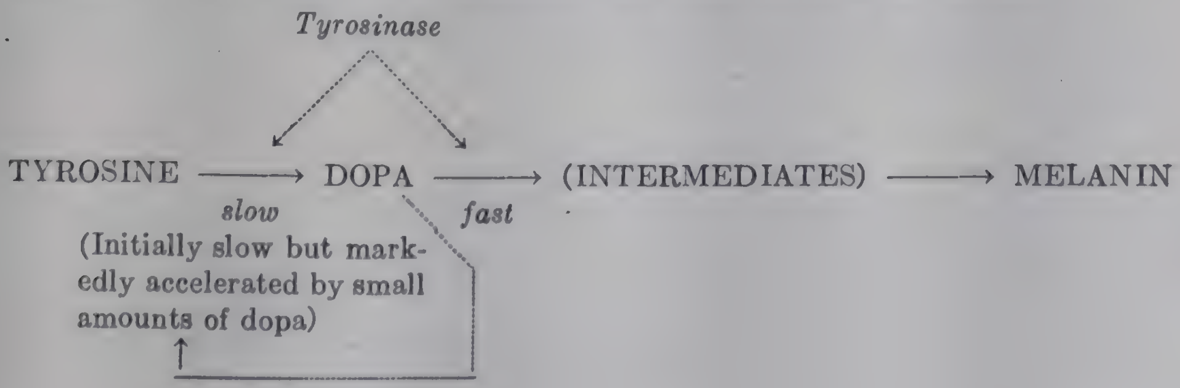
FIG. 4. The relation of dopa concentration to the induction period in the enzymatic oxidation of tyrosine by mouse melanoma preparations at pH 6.8 and 38°.

reaction had already been consumed. 0.02 ml. of the reaction mixtures and suitable controls of tyrosine and dopa were run in the usual manner on individual strips of filter paper (Whatman No. 1) 1.3 cm. in width. After the chromatogram had been allowed to develop for 5 hours at room temperature, the filter paper strips were dried and sprayed with ninhydrin solution. Ninhydrin gives a red color with tyrosine and a reddish brown color with dopa.

With the control solutions, tyrosine was found to concentrate at 9 to 10 cm. from the starting point and dopa at 5 to 7 cm. No dopa could be detected in the 0, 15, and 30 minute specimens. The chromatogram from the 50 minute sample had a definite reddish brown spot at 5 to 7 cm. from the starting point, and it seemed to be identical in position and color (but less intense) with the dopa controls. Unchanged tyrosine was evidenced by a typical red spot 2 to 3 cm. further along the filter paper strip.

DISCUSSION

In the past mammalian tyrosinase and dopa oxidase have been considered to be distinct enzymes. The work reported here indicates that there is no necessity for postulating the presence of separate enzymes for these two activities. Preparations from the Harding-Passey mouse melanoma catalyze the oxidation of both tyrosine and dopa to melanin. Dopa, in addition to being oxidized to melanin, serves to diminish the induction period in the enzymatic oxidation of tyrosine. We were not able to separate tyrosinase and dopa oxidase activity, although it was possible to prepare fractions with long induction periods in the oxidation of tyrosine. Such fractions appear superficially to be free from tyrosinase activity; however, it was invariably possible to demonstrate that these fractions catalyze the oxidation of tyrosine rapidly and completely in the presence of small amounts of added dopa. For these reasons it is recommended that the separate terms *tyrosinase* and *dopa oxidase* be abandoned in favor of the single term *tyrosinase* to describe the enzyme (or enzyme complex) involved in the oxidation of both tyrosine and dopa to melanin. This concept is illustrated diagrammatically as follows:


Tyrosinase
TYROSINE
DOPA
(INTERMEDIATES)
MELANIN
slow
fast
(Initially slow but markedly accelerated by small amounts of dopa)

Differential centrifugation of extracts from the Harding-Passey mouse melanoma indicates that tyrosinase activity is associated with cellular fragments. Claude (12, 15) has shown that nuclei and mitochondria of cells from several different tissues can be removed as precipitates from tissue extracts by centrifugation at low speeds for 20 minutes and then at high speeds for 10 minutes. Prolonged centrifugation will separate small cytoplasmic particles known as microsomes. From our centrifugation data alone, it appears that the enzymatically active particles of our preparation are microsomes or particles the size of microsomes which were formerly parts of larger aggregates that were dispersed during the experiments.

At present little is definitely known concerning the non-dialyzable, heat-stable substance in the crude tumor extracts that shortens the induction period in the oxidation of tyrosine. The possibility that this substance is dopa bound in a polypeptide chain should be considered.

There are many similarities between tyrosinase from plant and mammalian sources. Enzyme preparations from both sources catalyze the oxidation of both tyrosine and dopa to melanin, and dopa shortens the induction period in the tyrosine-tyrosinase reaction. Dopa is formed by both types of enzymes when tyrosine is oxidized. Much work has been done to determine whether or not plant tyrosinase is one enzyme or a mixture of several enzymes. The comprehensive work of Dawson and Mallette (16) indicates that all known properties of potato enzyme preparations may be explained in terms of a single entity. As stated previously, the work reported here shows that no distinction can be made between mammalian tyrosinase and dopa oxidase.

Plant and mammalian tyrosinases differ in some respects. Mammalian tyrosinase is associated with the particulate matter of cells and plant tyrosinase is not. Mammalian tyrosinase does not appear to be as readily inactivated during the course of a reaction as does plant tyrosinase. No quantitative relation has been found between the concentration of dopa and the induction period in the plant tyrosinase-tyrosine reaction, but a definite relation between the negative logarithm of the dopa concentration and the induction period exists in the mammalian tyrosinase-tyrosine reaction.

SUMMARY

1. The preparation and properties of mammalian tyrosinase from the Harding-Passey mouse melanoma are presented. It is shown that the enzymatic activity is associated with cytoplasmic particles and is not in true solution, at least in the type of tissue extracts ordinarily prepared.

2. The enzymatic activities of these preparations against tyrosine and

dihydroxyphenyl-L-alanine (dopa) as substrates were studied. For the mammalian enzyme, it is shown that the prevalent distinction between tyrosinase and dopa oxidase is not valid under certain experimental conditions. It is recommended, therefore, that the term *tyrosinase* be used for this enzymatic activity instead of the separate terms *tyrosinase* and *dopa oxidase.*

3. Dopa shortens the induction period in the enzymatic oxidation of tyrosine. A quantitative relationship exists between the dopa concentration and the induction period.

4. Dopa is formed during the mammalian tyrosinase-tyrosine reaction. This was demonstrated by means of filter paper chromatography.

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RATE OF EXCRETION OF RADIOACTIVE SULFUR AND ITS CONCENTRATION IN SOME TISSUES OF THE RAT AFTER INTRAPERITONEAL ADMINISTRATION OF LABELED SODIUM SULFATE

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(Received for publication, November 15, 1948)

A study of the distribution of S^{35} in the rat, 14 to 16 hours after the intraperitoneal administration of sodium sulfate, labeled with S^{35}, has been reported by Singher and Marinelli (1). Of particular interest is their observation that the highest concentration of the radioactive sulfur was found in the bone marrow. This observation stimulated us to determine the change with time in the concentration of S^{35}, given as labeled sodium sulfate, in the bone marrow of the rat and to relate this changing concentration with the concentration in other tissues, particularly the blood.

Only a brief report (2) was found in the literature on the excretion in urine of S^{35} ingested as sodium sulfate. The rate of excretion of S^{35} in the urine and feces of rats after intraperitoneal injection of labeled sodium sulfate was, therefore, also determined.

EXPERIMENTAL

Adult rats, 180 to 330 gm. in weight, from the colony of Professor E. V. McCollum, were each given 1 mg. of sodium sulfate (in 1 ml. of distilled water), labeled with radioactive sulfur (S^{35}),[1] by intraperitoneal injection. They were then placed in individual metabolism cages, with food and water, to allow for the separate collection of urine and feces. The cages had been so designed by Professor McCollum and his coworkers that there was a minimum of food spillage, and therefore contamination, of urine and feces with food. The rats were sacrificed at intervals of time, as follows: A rat was anesthetized in an ether jar. The heart was exposed and as much blood as possible was withdrawn directly from the heart with a hypodermic needle and syringe. A portion of the liver and the brain were then removed and placed in small vials. Weighed samples of these tissues, the feces collected in the time the rat sojourned in the metabolism cage, and 1 ml. of the blood were placed in separate nickel crucibles, each of which contained 2 ml. of 10 per cent sodium hydroxide and 4 gm. of anhydrous sodium carbonate. The crucibles were placed in a drying oven at 110–120° until dry. The dry material in each crucible was oxidized with sodium peroxide according to Bailey (3).

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[1] The S^{35} used in this investigation was supplied by the Clinton Laboratories, Monsanto Chemical Company, and obtained on allocation from the United States Atomic Energy Commission.

The humeri, femurs, and tibiae were freed of muscle and periosteum. The epiphysis of each bone was cut off at its junction with the diaphysis. The diaphysis of each bone was then freed of its marrow by pushing the latter out with a stainless steel wire. This marrow was placed immediately in a small screw cap vial and weighed. The residual marrow in the bone was wiped out by pushing through a small plug of moist cotton repeatedly until no further marrow was in evidence. As a check, each bone was split and carefully examined. It was further cleaned, if necessary, by wiping with moist cotton. The bone shafts were combined and powdered in a stainless steel mortar with a stainless steel pestle. A weighed portion of the powdered bone was oxidized with Benedict-Denis reagent (4).

The weighed bone marrow was extracted 3 times with at least 10 times its weight of 5 per cent trichloroacetic acid in a centrifuge tube. The combined extracts and the residue were each neutralized with 10 per cent sodium hydroxide, with phenolphthalein as an indicator, and then oxidized with Benedict-Denis reagent (4).

The urine excreted by each rat was combined with the washings of the metabolism cage and diluted to a volume of 100 ml. The total sulfur in aliquots of the urine was oxidized to sulfate according to Denis (4).

Before precipitation of sulfate from any sample as barium sulfate, 5 ml. of 0.05 N sodium sulfate solution were added to each sample so as to bring the final weight of barium sulfate to about 30 mg. Precipitation of barium sulfate was allowed to proceed for 16 to 20 hours at room temperature.

All barium sulfate samples were isolated by centrifugation and, after washing with distilled water in the centrifuge tubes, transferred to counting cups as slurries in 70 per cent ethanol as previously described (5).

The activity of each sample was determined with mica end window (2.8 mg. per sq. cm.) Geiger-Müller tube (Victoreen) and a Cyclotron Specialties scaler. At least two separate determinations of the activity of each sample were made for a sufficiently long period of time to obtain a precision of about 2 per cent. All values were corrected for decay and self-absorption.

RESULTS AND DISCUSSION

Of the twenty-seven rats used, fourteen were males and thirteen were females. The points given in Figs. 1 to 3 are average values, calculated from results on four animals at 4 hours, three animals at 8 hours, four animals at 16 hours, eight animals at 24 hours, and two animals each at 48, 72, 96, and 120 hours.

Fig. 1 shows the rate at which the S^{35} was found to be excreted in the urine and feces of the rats. Approximately 67 per cent of the activity injected was excreted in the urine by the end of the 24th hour. Borsook *et al.* (2) could account for only 47 per cent at the end of 24 hours in the urine of a man. The subject studied by Borsook *et al.* ingested the labeled sodium sulfate by mouth. The rats studied by us received the sodium sulfate containing S^{35} by intraperitoneal injection. This difference in the route of administration may account for the difference in the fraction recovered at the end of 24 hours. On calculating the intake by the human subject and by the rats, assuming that the human subject weighed somewhere within the limits of 45 to 90 kilos, one

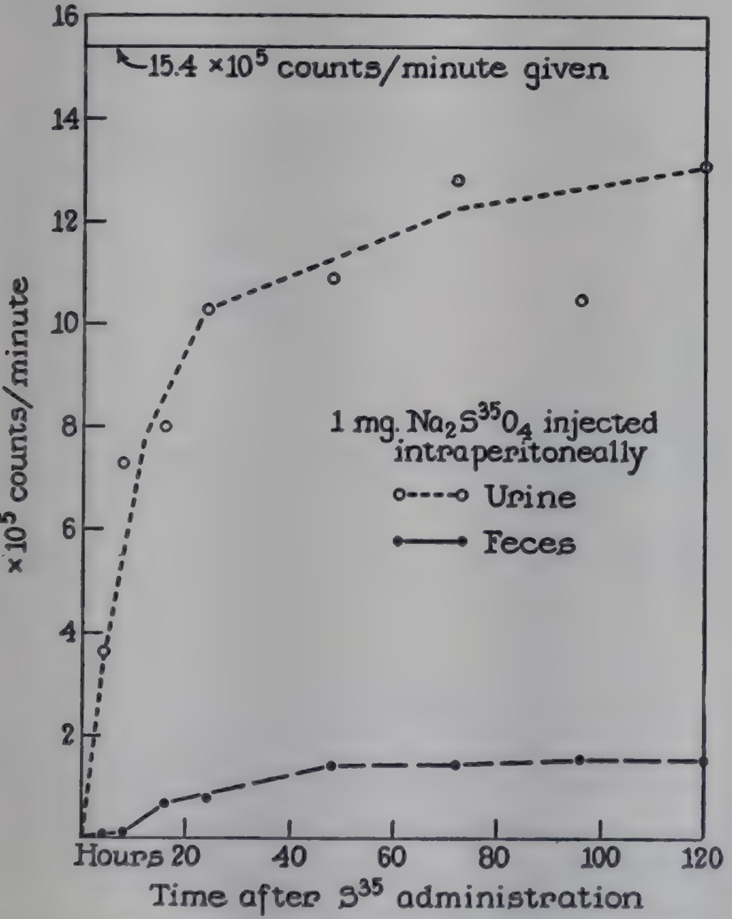

FIG. 1. Excretion of S^{35} in the urine and feces by adult rats after intraperitoneal injection of labeled sodium sulfate.

arrives at an intake of approximately 9.8 to 19.7 mg. of sodium sulfate per kilo as compared to approximately 4 mg. per kilo by the rats. This difference in intake might also be considered as a possible explanation for the difference in amount of S^{35} found excreted in the urine within 24 hours. One should, however, consider in addition a possible species difference as regards rate of sulfate excretion.

By the end of the 120th hour after the administration of the labeled sodium sulfate, the activity of S^{35} recovered in the urine accounted for approximately 85 per cent of the activity given. Approximately 95 per cent of the S^{35} is accounted for at the end of 120 hours if the activities found in the urine and feces are added.

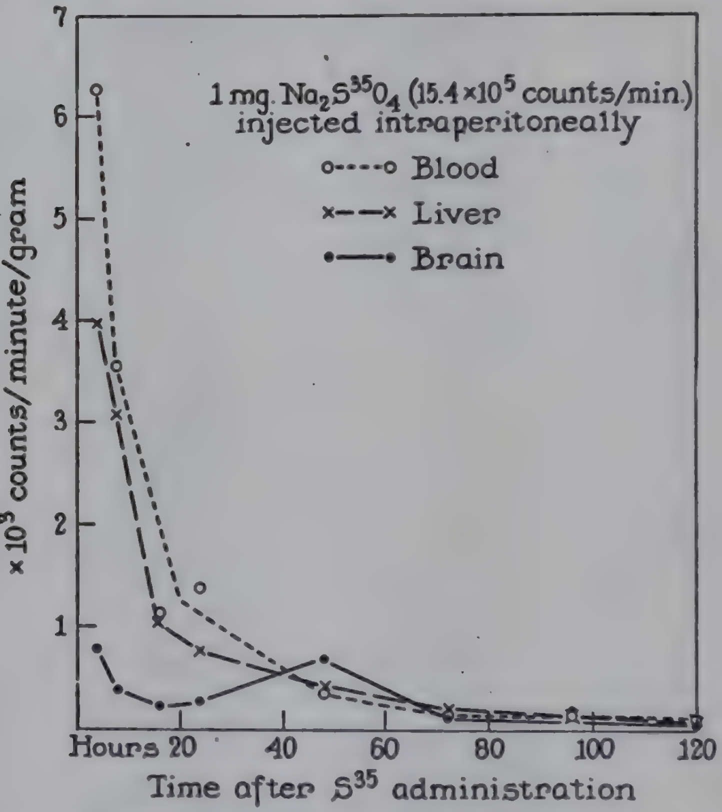

FIG. 2. Retention of S^{35} in the blood, liver, and brain by adult rats after intraperitoneal injection of labeled sodium sulfate.

In Fig. 2 are presented curves showing the change in concentration of S^{35} with time in the blood, liver, and brain. From these curves it would seem that sulfate sulfur as such, when it enters the circulation or the liver in a normal rat, is rapidly eliminated from these tissues. Whether the brain is similar in this respect or whether it slowly accumulates S^{35} presented to it as sulfate sulfur is difficult to say without extending the number of observations reported here. In any case, the concentration of S^{35} in the liver and blood appeared to have reached a similar concentration at about the 48th hour after the injection of the

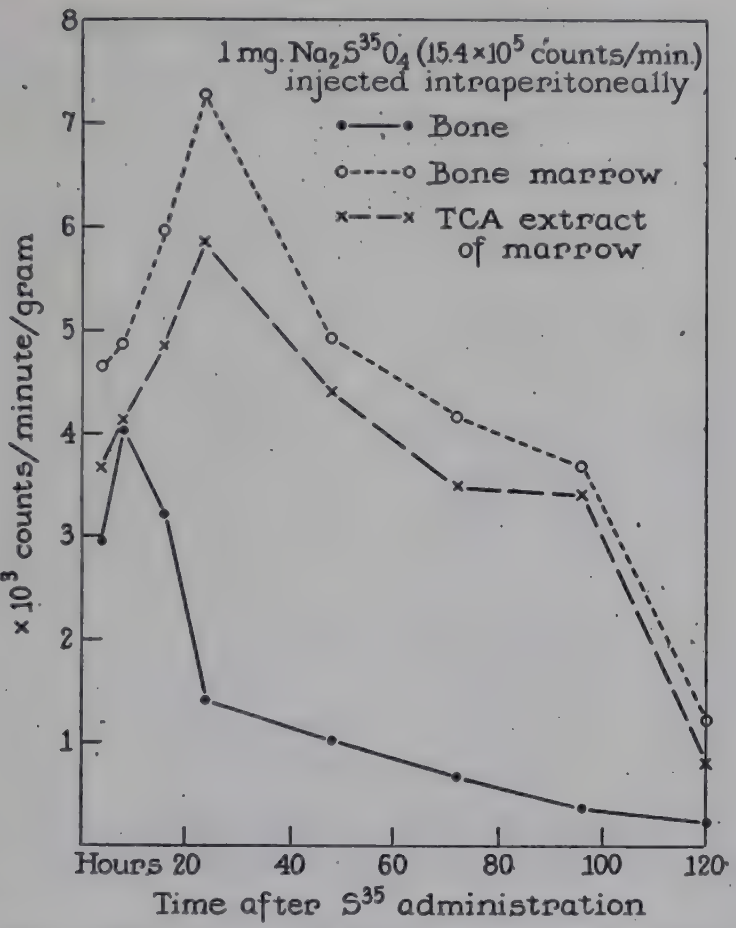

FIG. 3. Retention of S^{35} in the bone and bone marrow by adult rats after intraperitoneal injection of labeled sodium sulfate.

TABLE I

Extent of S^{35} Removal from Rat Bone Marrow by 3-Fold Extraction with 5 Per Cent Solution of Trichloroacetic Acid

A male rat, 355 gm. in weight, was used 24 hours after the intraperitoneal administration of 1 mg. of sodium sulfate, containing S^{35} (15.4 × 10^5 counts per minute). The values for activity are corrected for radioactive decay and self-absorption.

Sample	
	counts per min. per gm.
1st three trichloroacetic acid extracts combined	4960
4th trichloroacetic acid extract	0–10
Three alcohol-ether (1:3) extracts combined	64
Residue	793

labeled sulfate. A similarity in the concentration of S^{35} in the brain and blood, from the observations thus far made, was more slowly attained; a similar concentration was reached at about the 72nd hour.

In contrast to the rapid fall of the S^{35} concentration in blood and liver, after intraperitoneal injection of labeled sodium sulfate, is the pronounced rise in the concentration of S^{35} found in bone and bone marrow (Fig. 3). The highest concentration in the bone was observed at about the 8th hour, that in the bone marrow at about the 24th hour after injection of the labeled sodium sulfate. The subsequent drop in the S^{35} concentration in these tissues is also less rapid, particularly in the bone marrow. Even at the end of 120 hours, on a weight basis, the S^{35} concentration is about 2 times as high in the bone and nearly 12 times as high in the bone marrow as in the blood. The observations confirm the report of Singher and Marinelli (1) that the concentration of S^{35}, after administration of labeled sodium sulfate, is higher in bone marrow than that found in most of the other tissues of the rat. The major portion of the activity found in the bone marrow is in a compound or compounds which are soluble in a 5 per cent solution of trichloroacetic acid. That a 3-fold extraction of bone marrow with a 5 per cent solution of trichloroacetic acid, as employed, was effective in removing all or nearly all of the S^{35}-containing material, which was soluble in this solution, is indicated in Table I. Further work on the characterization of the materials containing S^{35} in the various fractions listed in Table I is contemplated.

SUMMARY

The excretion by rats in urine and feces of S^{35} given in the form of sodium sulfate appears to be rapid. Excretion by these routes accounts for the major portion of the S^{35} given. By the end of the 120th hour approximately 95 per cent was found to have been eliminated by these routes.

In the period of 120 hours the concentration of S^{35} in the blood, liver, and brain was found to have fallen to relatively low levels. A similar concentration of S^{35} in the blood and liver was attained by the 48th hour, in the blood and brain by the 72nd hour.

In contrast to the rapid fall observed in the liver and blood, the concentration of the S^{35} was found to increase until about the 8th hour in bone and until about the 24th hour in bone marrow. The subsequent fall in the S^{35} concentration of the bone and bone marrow was also slower than that in blood, liver, and brain.

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ULTRACENTRIFUGATION OF HYPOPHYSEAL GROWTH HORMONE*

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(Received publication, October 13, 1948)

The isolation of the growth hormone from ox pituitaries has recently been described (1). It was found that the preparation behaves as a single component in diffusion, electrophoresis, and solubility experiments (1, 2). For further characterization of the hormone ultracentrifugal experiments have been made and the results are reported here.

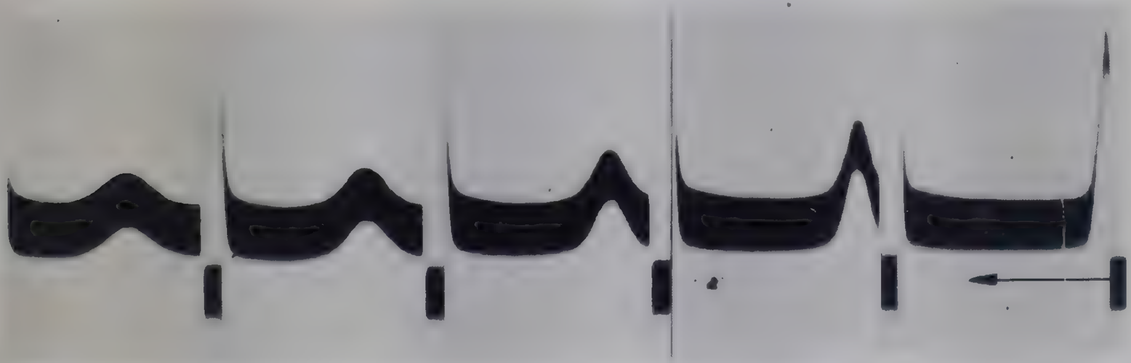

FIG. 1. The Svensson schlieren patterns of hypophyseal growth hormone, taken at 1920 second intervals during sedimentation at 165,000 × g in an ultracentrifuge.

Sedimentation velocity was measured in a Spinco ultracentrifuge[1] operating at 165,000 × g. The hormone solutions (0.5 per cent) were prepared with borate buffer of pH 9.7 or saline at pH 9.1. Table I presents a typical run in a borate buffer; the sedimentation velocity constant was calculated by the procedure described by Svedberg and Pedersen (3). It may be noted that the individual values of s_{20} calculated at 1920 second intervals agree to within 3 per cent. The Svensson schlieren diagrams shown in Fig. 1 indicate that the hormone is essentially monodisperse. As shown in Table II, the average sedimentation constant, corrected as

* Aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council, the United States Public Health Service, contract No. RG-409, and the Research Board of the University of California, Berkeley, California.

[1] Manufactured by the Specialized Instruments Corporation, Belmont, California. We wish to thank Dr. M. Calvin for his kindness in putting the instrument at our disposal.

if occurring in pure water at 20°, from three experiments in two different solvents, was $s_{20} = 3.1 \times 10^{-13}$ cm. per second per unit field.

From the sedimentation constant, s_{20}, and the diffusion constant, D, the molecular weight, M, may be calculated by the familiar equation, $M = RTs/D(1-V\rho)$, where R is the gas constant, T the absolute temperature, V the partial specific volume, and ρ the density of the solvent. Using the earlier determinations of D and V (2), we have computed the molecular weight of the growth hormone to be 44,000, which is in fair agreement with that obtained by other methods (Table III).

TABLE I

Calculation of Sedimentation Constant of Growth Hormone

Protein concentration, 5.0 mg. per cc.; time intervals between exposures, 1920 seconds; temperature, 27.1°; solvent, borate buffer, pH 9.7.

Observation No.	Δx	x_m	$x\omega^2(10^{-7})$	$s \times 10^{13}$
2	0.113	6.01	16.0	3.7
3	0.110	6.12	16.3	3.5
4	0.119	6.24	16.6	3.7
5	0.114	6.35	17.0	3.5

* $s_{av.} = 3.6 \times 10^{-13}$; $s_{20} = s_{av.} \times 0.85 = 3.1 \times 10^{-13}$.

TABLE II

Sedimentation Constant of Growth Hormone Solutions

Protein concentration, 5.0 mg. per cc.

Experiment No.	Solvent	Temperature	$s \times 10^{13}$	$s_{20} \times 10^{13}$
		°C.		
II	Borate buffer, pH 9.7	27.1	3.6	3.1
III	" " " 9.7	24.6	3.5	3.2
IV	Saline, pH 9.1	28.2	3.6	3.0
Average				3.1

TABLE III

*Molecular Weight of Growth Hormone by Different Methods**

Method	Mol. wt.
Osmotic pressures	44,250
Minimum by analysis	47,300
Diffusion-viscosity	39,300
Ultracentrifugation	44,000

* For other values, see Li (4).

SUMMARY

The anterior hypophyseal growth hormone isolated from ox pituitaries was found to be essentially monodisperse in the ultracentrifuge and had a sedimentation constant, $s_{20} = 3.1 \times 10^{-13}$ cm. per second per unit field. The molecular weight was calculated to be 44,000.

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THE USE OF AMINIZED AND PHOSPHORYLATED COTTON FABRICS AS ION EXCHANGE MATERIALS IN THE PREPARATION OF OIL SEED PROTEINS

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Protein from oil seed meal is usually prepared by dispersing the protein at a pH more basic than its isoelectric point, separating the undispersed material, and precipitating the protein by lowering the pH to about the isoelectric point. The increase in pH is ordinarily accomplished by addition of base to the meal suspension, while lowering of the pH is effected by addition of acid, frequently sulfurous acid, or by the "self-souring process" in which acid is produced by microorganisms. The work to be reported shows that the required shifts in pH may be accomplished by use of ion exchange materials and that ion exchange materials prepared from cotton fabric are particularly convenient for this purpose. Protein preparations made in this way are low in ash and phosphorus content because of removal of ions in the process.

EXPERIMENTAL

Most of the experiments were made on peanut meal produced by extracting ground, red skin, Spanish type peanut kernels with commercial pentane in a large Soxhlet extractor. A few were made on solvent-extracted cottonseed meal prepared by a process which removes most of the pigment glands (1). Another starting material was a sample of crude peanut protein that had been prepared from solvent-extracted meal from red skin peanuts as previously described (2). It contained 16.65 per cent nitrogen, 1.71 per cent ash, and 0.53 per cent phosphorus on the dry weight basis.

The anion exchange materials used to increase the pH of the meal suspensions were aminized cotton fabric[1] (3), Amberlite IR-4-B, and De-Acidite.[2] They were regenerated by treatment with 5 per cent sodium hydroxide solution for 10 minutes. The excess alkali was removed by washing with successive portions of distilled water, allowing each portion to remain in contact with the exchange material for several minutes. This washing was continued until the pH of the wash water was less than 6.0.

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[1] This material may be prepared by wetting cotton fabric with a solution containing 25 per cent sodium hydroxide, 10 per cent 2-aminoethylsulfuric acid, and 65 per cent water, heating in a drying oven for 40 minutes at 100°, and washing thoroughly.

[2] The mention of trade products does not imply that they are endorsed or recommended by the Department of Agriculture over similar products not mentioned.

The cation exchange material used to reduce the pH of the protein dispersions in most of the experiments was phosphorylated cotton fabric[3] prepared by the urea-phosphoric acid method (4). Its use as an ion exchange material has been previously investigated (5). Amberite IR-100 and Zeo-Karb H were also used, but it was necessary to pack these in a suitable length of Visking casing. Owing to the enclosure in this membrane, about 40 hours with mechanical stirring or 4 to 5 days without stirring were required to effect the necessary pH change, while with phosphorylated cloth placed directly in the dispersion the maximum effect was obtained in about 20 minutes. The materials were regenerated by treating them with 5 per cent hydrochloric acid solution for 10 minutes, and washing repeatedly with distilled water until the pH of the washings was above 3.0. The complete removal of excess acid was further verified by development of a pink color on addition of a drop of phenolphthalein indicator and a drop of 0.1 N sodium hydroxide solution to 50 ml. of the wash water.

In a typical experiment, 100 gm. of solvent-extracted peanut meal were suspended in 900 ml. of water with mechanical stirring. The pH of the suspension was 6.60. A piece of regenerated aminized cotton fabric (0.41 per cent nitrogen), weighing 18 gm., was added and mechanical stirring continued for 20 minutes, at which time the pH had increased to a constant value of 6.95. The fabric was freed of as much of the suspension as possible and replaced with a 20 gm. piece of fabric. After stirring for 20 minutes the pH had risen to 7.48, when the material was replaced with an 18 gm. piece of fabric which raised the pH to 7.80. After the last piece of fabric was removed, the meal suspension was centrifuged and the supernatant liquid filtered. The filtrate was then treated with successive pieces of phosphorylated cotton fabric (4.6 per cent phosphorus) while stirring until the pH was reduced to 4.5. As each piece of fabric was removed from the protein dispersion, it was squeezed and then rinsed in water to remove as much of the adhering protein as possible. A total of 25 gm. (five 5 gm. pieces) of phosphorylated fabric was required. After the last piece of fabric had been removed, the precipitated protein was separated by centrifugation, washed once with water, twice with ethanol, and dried in a vacuum oven at 40°. The yield of protein with the fabrics was 29 per cent of the weight of the meal, while a control preparation made with sodium hydroxide and sulfur dioxide yielded 32 per cent.

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[3] This material may be prepared by wetting cotton fabric with a solution containing 49.6 per cent urea, 18.4 per cent phosphoric acid, and 32 per cent water, squeezing out the excess solution, heating the fabric for 30 minutes at 150°, and washing thoroughly.

The dispersion of protein from cottonseed meal requires a considerably higher pH than does that from peanut meal. The highest pH obtainable with cottonseed meal suspensions was 9.0 with aminized cotton fabric. Consequently, in preparing protein from cottonseed meal, the residue from the treatment with aminized fabric was dispersed further with dilute sodium hydroxide to produce an additional fraction. Since the pH values obtained with Amberlite IR-4-B or De-Acidite were considerably lower, these materials were unsatisfactory for the preparation of cottonseed protein.

TABLE I

Per Cent Ash, Phosphorus, and Nitrogen of Protein Prepared from Peanut Meal

Method of dispersion	Method of precipitation					
	Sulfur dioxide			Phosphorylated fabric		
	Ash	P	N	Ash	P	N
Sodium hydroxide, pH 7.9	0.41	0.40	17.3	0.33	0.40	17.1
Aminized fabric " 7.8	0.17	0.13	17.5	0.22	0.20	17.5
" " *				0.09	0.12	18.1
" " †				0.13	0.09	17.1
De-Acidite, pH 7.5	0.24	0.18	17.1	0.23	0.22	16.9
Amberlite IR-100, pH 7.5	0.34	0.43	16.9	0.40	0.48	17.1

* pH 8.0 with aminized fabric, to pH 4.1 with phosphorylated fabric, and back to pH 4.5 with aminized fabric.

† pH 8.5 with aminized fabric, to pH 3.9 with phosphorylated fabric, then to pH 6.65 with aminized fabric, and back to pH 4.5 with phosphorylated fabric.

Before analysis, the dried proteins were equilibrated in the air and ground to a fine powder. Moisture was determined by drying for 2 hours in a forced draft oven at 130°. To obtain ash values, samples were charred at 250° and ignited at 600° for 4 hours in a pyrometer-controlled electric muffle. Phosphorus was determined by the reduced molybdate method after digestion with sulfuric acid and hydrogen peroxide (6). Nitrogen determinations were made by the Kjeldahl method with mercury as a catalyst. All values given in Tables I to III are on a moisture-free basis.

RESULTS AND DISCUSSION

Analytical results on several samples of protein prepared in different ways from solvent-extracted peanut meal are shown in Table I. The commercial anion exchange resins did not raise the pH to the desired value of 7.8, but the proteins made with De-Acidite were lower in ash and phosphorus than the control proteins dispersed with sodium hydroxide. Aminized cotton fabric was quite adequate in increasing the pH of the meal suspension and produced protein preparations very low in ash and phosphorus and high in nitrogen.

Similar results are shown in Table II for preparations made by redispersing and reprecipitating a crude sample of peanut protein. In these experiments the aminized fabric increased the pH to only 6.6, owing to removal of nearly all the anions. The dispersion of the protein remaining in the residues was completed by adding dilute sodium hydroxide solution to obtain pH 7.8. The preparations made with the ion exchange fabrics were very low in ash and phosphorus and high in nitrogen.

TABLE II

Analysis of Samples Made from Crude Peanut Protein

Dispersion		Precipitation		Analytical values		
Agent	pH	Agent	pH	Ash	P	N
				per cent	*per cent*	*per cent*
NaOH	6.6	Sulfur dioxide	4.4	0.74	0.82	17.3
Residue from above, NaOH	7.8	" "	4.5	0.63	0.38	16.9
Aminized fabric	6.6	" "	4.3	0.14	0.06	17.9
Residue from above, NaOH	7.8	" "	4.5	0.18	0.07	17.8
NaOH	6.6	Phosphorylated fabric	4.5	0.64	0.76	17.4
Residue from above, NaOH	7.8	" "	4.4	0.33	0.56	16.9
Aminized fabric	6.6	" "	4.5	0.08	0.04	18.3
Residue from above, NaOH	7.8	" "	4.4	0.10	0.09	18.5

Results obtained with cottonseed meal are given in Table III. The ash and phosphorus contents of the preparations of cottonseed protein made by use of the ion exchange fabrics were higher than in the corresponding preparations from peanuts, but were lower than in cottonseed protein preparations made with sodium hydroxide and sulfur dioxide.

Aminized cotton fabric was the most satisfactory anion exchange material for the preparation of protein, since a higher pH could be obtained with it and the ash and phosphorus content of the preparations were lower than that obtained with either of the commercial anion exchange resins. Phosphorylated cotton fabric was the most rapid and conveniently used cation exchange material because it did not have to be enclosed in a membrane. All of the ion exchangers gave satisfactory yields of protein, although somewhat smaller than were obtained with sodium hydroxide and sulfur dioxide.

Considerable quantities of yellowish brown-colored substances attached themselves to the aminized fabric during the dispersion of both peanut and cottonseed protein. Some of these substances were removed on regeneration of the fabric, but others remained fixed on the cloth. It seemed possible that this removal of colored substances by the aminized cloth would improve the color of solutions of protein so prepared. However, alkaline solutions of peanut protein prepared by means of aminized cotton fabric were as dark as those from proteins prepared with sodium hydroxide.

TABLE III

Analysis of Protein Preparations Made from Cottonseed Meal

Dispersion		Precipitation		Analytical values		
Agent	pH	Agent	pH	Ash	P	N
				per cent	*per cent*	*per cent*
NaOH	9.0	Sulfur dioxide	4.1	2.60	1.14	15.8
Residue from above, NaOH	10.5	" "	4.4	0.93	0.62	17.0
Aminized fabric	9.0	" "	4.1	1.21	0.99	13.7
Residue from above, NaOH	10.5	" "	4.4	0.86	0.65	16.2
NaOH	9.0	Phosphorylated fabric	4.1	1.99	1.09	16.0
Residue from above, NaOH	10.5	" "	4.5	0.55	0.89	16.1
Aminized fabric	9.0	" "	4.2	0.88	0.69	13.6
Residue from above, NaOH	10.5	" "	4.4	0.13	0.35	18.0

The low phosphorus content of proteins prepared by use of aminized fabric indicated that the phosphorus compounds were combining with the fabric. This was verified by analyzing a piece of aminized fabric which had been used to disperse crude peanut protein and subsequently thoroughly washed. It was found to contain 0.29 per cent phosphorus, while a piece of fabric that had not been aminized but exposed in the same way to crude peanut protein contained no phosphorus. Aminized fabric which has not been used for the preparation of protein does not contain any phosphorus.

We are indebted to Vidabelle O. Cirino and Harry E. Conrad for the nitrogen and phosphorus determinations.

SUMMARY

Protein preparations low in ash and phosphorus content may be made by the use of anion exchange materials to increase the pH of oil seed meal suspensions and cation exchange materials to reduce the pH of the protein dispersion to the isoelectric range. Preparations very low in ash and phosphorus may be obtained by the alternate use of aminized cotton fabric and phosphorylated cotton fabric until deionization is almost complete prior to separation of the protein curd. The method is also useful for the purification of protein preparations made by the usual methods. While commercial ion exchange resins may also be used, the results with the fabrics have been better. The fabrics are also more convenient and are readily recovered and regenerated.

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PREPARATION OF RADIOACTIVE IODOCASEIN*

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(Received for publication, September 2, 1948)

We have recently prepared iodocasein containing radioactive iodine (I^{131}) for use in studies, to be described in detail elsewhere, on the metabolism of calorigenic substances in human beings. The procedure followed was, in general, that of Reineke and Turner (1, 2) except for modifications necessitated by the incorporation and handling of I^{131}. These modifications shorten the procedure somewhat and aid in obtaining conveniently a product having sufficient specific activity and biologic potency. It is the purpose of the present paper to describe the preparation and properties of the radioactive iodocasein so obtained.[1]

Methods

Chemical Analyses. Tyrosine—After hydrolysis of the protein according to the method of Bhagvat and Sreeramamurthy (4), tyrosine was determined by the method of Folin and Marenzi (5).

Iodine—The method of Shahrokh (6) was used with the following changes: (1) In lieu of the transfers originally described, the entire procedure was carried out in a 100 ml. Kjeldahl flask marked at a 15 ml. level. (2) Phenol, which is added just prior to titration, was omitted, since its use led to lower iodine values. (3) Glass beads were substituted for pumice, leading to a clearer and sharper end-point.

Thyroxine—Hydrolysis with barium hydroxide and distribution of thyroxine in butanol was effected according to the procedure of Roche and Michel (7). The purified butanol extract was analyzed for iodine, as was done by Reineke and coworkers (8) in their studies of thyroxine in iodinated casein, and the resulting value was converted to thyroxine by means of the factor 1.529.

Biologic Analysis—The methods of Deanesly and Parkes (9) and of Hamilton, Albert, and Power (10) were employed.

Radiologic Analyses—0.2 ml. aliquots of solutions containing radioactive iodine were dried on copper planchettes with 0.1 ml. of silver nitrate

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* Abridgment of part of a thesis submitted by Dr. Hamilton to the Faculty of the Graduate School of the University of Minnesota in partial fulfilment of the requirements for the degree of Master of Science in Medicine.

[1] After completion of our work, Frieden, Lipsett, and Winzler (3) also reported the use of the method of Reineke and Turner (1, 2) for the preparation of radioiodocasein.

containing 1 mg. of silver per ml., and counted with a β-ray Geiger coun
in connection with a scaling circuit (Autoscaler, Tracerlab, Inc., Bost
Massachusetts). Counts were corrected for decay and self-absorpti
when necessary, and expressed usually in terms of per cent of radioactiv
originally involved in a given experiment or operation, by means of co
parison with a simultaneously counted pilot of the sample of I^{131} used.

Reagents—Borden's Labco (vitamin-free) casein containing 4.9 per ce
tyrosine was used. I^{131} was obtained from the Clinton Laboratories, O
Ridge, Tennessee, in carrier-free solution. The solution was adjusted
pH 8.0, calcium chloride was added to precipitate oxalate, sodium iodi
was added as carrier, and the solution was diluted so that 1 ml. containe
γ of sodium iodide and 500 microcuries of I^{131}. Other reagents used we

TABLE I

Summary of Six Preparations of Radioactive Iodocasein

Preparation	Amounts used		Analyses of product					
	I^{131}	Casein	Total organic I^{127}	Thyroxine	Per cent thyroxine I^{127} of total I^{127}	Per cent thyroxine I^{131} of total I^{131}	I^{131}, per cent of I^{131} used	Speci activi
	microcuries	*gm.*	*per cent*	*per cent*				*microcu per mg*
I	100	2	5.2	2.0	26	31	29	0.02
J	186	1	4.9	2.5	33	45	35	0.06
K	260	1	5.4	2.8	34	37	28	0.08
L	150	1	6.0	2.6	29	22	28	0.05
M	6000	0.5	5.0	3.0	38	52	24	2.60
N	6000	0.5	6.3	3.3	35	34	38	3.60
Average			5.5	2.7	33	37	30	

sodium bicarbonate, 10 per cent nitric acid, 10 per cent sodium nitri
0.2 M acetate buffer at pH 4.6, aldehyde-free ethyl alcohol, and colloid
manganese oxides prepared according to the method of Friedemann a
Kendall (11).

EXPERIMENTAL

Sufficient sodium iodide to provide 4 atoms of iodine per mole of ty
sine plus an excess of 30 per cent to allow for loss in the liberation of iodi
was dissolved in 5 ml. of water containing the desired amount of I^{131} (va
ing from 100 to 6000 microcuries). The solution was placed in a 50 r
iced centrifuge tube shielded by lead bricks. Iodine was liberated in t
usual manner by nitric acid and sodium nitrite. After centrifugation

he cold, the iodine crystals were washed twice with 5 ml. of ice-cold water. o the elementary iodine was added a 2.5 per cent solution of casein in 1 er cent sodium bicarbonate containing 10 ml. of colloidal oxides of manganese per gm. of casein. The mixture was agitated in a cradle rocker at 0° for 18 to 20 hours. After incubation, the mixture was adjusted to H 4.6 with hydrochloric acid and centrifuged. The precipitate was ashed twice with acetate buffer at pH 4.6 and once with alcohol, and was ried. The entire procedure was carried out within 24 hours.

TABLE II

Characteristics and Comparison of Iodocaseins with Desiccated Thyroid

Preparation	Total organic I^{127}	Thyroxine	Per cent thyroxine I^{127} of total I^{127}	Average biologic activity, m.e.d.*
	per cent	*per cent*		*mg.*
odocasein (Protamone)†	5.8	3.3	36	0.4
" (Preparation C)‡	6.2	2.5	26	0.5
" (" M)§	5.0	3.0	38	0.2
" (" N)§	6.3	3.3	35	0.3
esiccated thyroid (strong)‖	1.0	0.4	27	0.4

* Median effective dose.

† Cerophyl Laboratories, Inc., Kansas City, Missouri.

‡ Non-radioactive, prepared as in the text.

§ Radioactive, *cf.* Table I.

‖ Parke, Davis and Company, Detroit, Michigan.

Results

Six preparations were made. The pertinent data are shown in Table I. osses of radioactivity were determined at each step in the procedure. he over-all recovery of I^{131} in the final dried product averaged 30 per ent. The incubation mixture contained 70 to 85 per cent of the original ctivity, indicating a 15 to 30 per cent loss during the preparation of free dine, its addition to the casein solution, and the period of incubation at 0°. Only a small portion of this loss, however, was found by direct estiation. The combined supernatant and buffer washes of the isoelectric recipitate accounted for a loss of 46 per cent and the alcohol wash acounted for a loss of 1.5 per cent. One batch was prepared by the original ethod of Reineke and Turner (2) which utilizes prolonged dialysis of the acubation mixture. The yield of I^{131} was not different from that deribed, indicating that the isoelectric procedure is as effective in removing cluded iodide as the dialysis method and has the advantage of conuming less time.

The average content of total organic iodine of the iodocasein was 5.5 per cent and the thyroxine content was 2.7 per cent. The proportion of thyroxine I^{127} to total I^{127} was 33 per cent, with which the radioactive analyses agreed well (37 per cent of the total I^{131} was thyroxine I^{131}). The specific activity of the compound, of course, varied with the amount of both I^{131} and casein used, and can be, therefore, altered to suit the particular purpose in mind. For studies on human subjects a physiologic dose of iodocasein was calculated to be 60 mg. per day on the basis of equivalence of physiologic action as compared with desiccated thyroid. Since 100 to 200 microcuries at the time of administration in man can be followed conveniently by present counting equipment, a preparation containing 2 to 3 microcuries per mg. was considered optimal for human subjects. This can be readily achieved in any reasonable amount with 12 millicuries of I^{131} per gm. of casein.

A comparison of the properties of two preparations of radioiodocasein of highest specific activity, a single non-radioactive preparation, a commercial brand (Protamone) prepared according to the method of Reineke and Turner (2), and desiccated thyroid is given in Table II. The total iodine and thyroxine of all iodocaseins are similar, as is their biologic activity. Desiccated thyroid, however, while containing about the same ratio of thyroxine iodine but much less total iodine, was found to be slightly less than half as active as the artificial iodoproteins.

SUMMARY

A procedure for the preparation of radioactive iodocasein of sufficient radioactivity and biologic activity for use in physiologic amounts in human metabolic studies is described.

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THE EFFECT OF ZINC-HYDROCHLORIC ACID HYDROLYSIS ON THE ESTROGENS IN HUMAN URINE*

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(Received for publication, September 8, 1948)

Smith and Smith (1) reported that boiling of human female urine for 3 hours with 4 gm. per cent of zinc dust and 15 volumes per cent of hydrochloric acid resulted in a marked increase of estrogen potency over that acquired by optimum conditions of simple acid hydrolysis; namely, 10 minutes boiling with 15 volumes per cent of hydrochloric acid. They were unable to account for all of the increased estrogenic potency by conversion of estrone to α-estradiol and more complete hydrolysis and they therefore postulated the occurrence in human urine of certain non-estrogenic compounds closely related to the estrogens (estrone, α-estradiol, and estriol), which were rendered estrogenic by hydrogenation (2). Pincus and Pearlman (3) have obtained evidence for the presence of an additional estrogen in human pregnancy urine which is ketonic and contains a hydroxyl group other than the usual phenolic one. The same type of estrogen recently was reported (4) as being present in human and rabbit blood. The synthesis of two more highly oxygenated estrogens related to estrone and α-estradiol has been accomplished (5), and the suggestion has been made (6) that these (16-ketoestrone and 16-keto-α-estradiol) may be the compounds postulated by Smith and Smith. Since previous work (7) from this laboratory has given some chemical (colorimetric) confirmation to the hypothesis of Smith and Smith, it was felt that further information on the effects of the hydrolytic technique on the known natural estrogens and on the newly synthesized estrogens might further our knowledge concerning the possible presence of additional estrogens in the urine.

We now present data on the effects of the hydrolytic process as derived by our procedure for the fractionation and photometric estimation of urinary estrogens (8) which show that zinc-hydrochloric acid hydrolysis of the estrogens in human urine (*a*) converts the major portion of estrone to estradiol, (*b*) yields estradiol titers which are higher than can be accounted for by mere conversion of estrone to estradiol, and (*c*) yields estriol titers

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which appear higher than can be accounted for by more complete hydrolysis. The effect of hydrolysis on known natural estrogens and on one of their conjugates (sodium estrone sulfate) does not satisfactorily account for these observations. The data derived from the addition of 16-keto-estrone and 16-keto-α-estradiol to estrogen-free (male) urine before hydrolysis appear to offer a satisfactory explanation.

EXPERIMENTAL

Collection and Preservation of Urine—The human pregnancy urine was collected over a 24 hour period without preservative and was utilized on the following day. If pooled human pregnancy urine or pooled human male urine was required, collection and storage was made with toluene.

Hydrolysis and Extraction—The urine was extracted, chromatographed, and the titer determined as previously described (8), except that the butyl alcohol extraction was omitted and the hydrolyzed urine was extracted with an ethyl ether-toluene mixture (9:1). This extract was concentrated to a convenient volume and separated into neutral and phenolic fractions (8). Wherever the *L*-520 mμ to *L*-420 mμ ratio of our Kober color product fell below 6.0, our color correction equation (9) was utilized.

TABLE I

Estrogen Titer of Human Pregnancy Urine

	Aliquot A (400 ml.), 10 min. refluxing, 15 vol. per cent HCl added	Aliquot B (400 ml.), 3 hrs. refluxing, 15 vol. per cent HCl + 4 gm. per cent zinc dust added	$\frac{\text{Aliquot B}}{\text{Aliquot A}}$
	γ	γ	
Estrone	340	35	
Estradiol	210	1180	
Estrone + estradiol	550	1215	2.20
Estriol	3200	5800	1.81
Estrone + estradiol + estriol	3750	7015	1.87

RESULTS AND DISCUSSION

The typical colorometric estrogen data on human pregnancy urine as derived by simple hydrochloric acid hydrolysis and the zinc-hydrochloric acid technique of Smith and Smith are summarized in Table I. Each urine aliquot after hydrolysis was processed in identical fashion, so that any difference in estrogen titer must be attributed to the hydrolytic techniques under investigation. Zinc-hydrochloric acid hydrolysis of human pregnancy urine caused almost complete depletion of the estrogen in the estrone fraction and yielded an estrogen titer in the estradiol fraction (calculated as α-estradiol) which was 2.2 times as much as the combined estrone and estradiol fractions by simple hydrolysis. The absence of estrone from the estradiol fraction was verified with the Zimmermann test (10). It is obvious that factors other than merely conversion of estrone to estradiol are involved when the zinc-hydrochloric acid hydrolytic technique of Smith and Smith is employed.

In order to test the hypothesis that more complete hydrolysis may account for the augmented estrogen titers obtained by zinc-hydrochloric acid hydrolysis, a number of experiments were performed in which the residual urine after simple hydrochloric acid hydrolysis and ether extraction was rehydrolyzed by the zinc-hydrochloric acid technique. Data typical of these experiments are summarized in Table II. In this experiment, a preliminary butyl alcohol extraction of the unhydrolyzed urine was performed as described in a previous publication (8), and the hydrolytic techniques were performed on aliquots of the aqueous extract of the residue from evaporation of the butyl alcohol. The Zimmermann test on the estrone fraction of Aliquot A gave a titer of 96 γ. The significantly higher titer of 220 γ obtained with the Kober reagent would suggest that the estrone fraction is contaminated with estradiol, presumably due to the limitations of the chromatographic fractionation procedure, in the presence of relatively large amounts of estradiol. Rehydrolysis by the zinc-hydrochloric acid technique of the urine extract (Aliquot B) following simple hydrochloric acid hydrolysis and extraction yielded an estrogen titer in the combined estrone-estradiol fractions even larger than

TABLE II

Estrogen Titer after Butanol Extraction of 24 Hour Urine Specimen and Hydrolysis of Aliquots of Extract

Aliquot	Conditions of hydrolysis	Estrogen titer			
		Estrone	Estradiol	Estrone + estradiol	Estriol
		γ	γ	γ	γ
A	15 vol. % HCl + 4 gm. % zinc	220	1500	1720	8700
B_1	15 " % " (10 min. refluxing)	360	250	610	5000
B_2	B_1 rehydrolyzed with zinc	46	640	686	1000
	Total estrogen titer, $B_1 + B_2$	406	890	1296	6000
	$\frac{B_1 + B_2}{A}$, %			76	69

those obtained by the initial simple hydrolysis. However, the combined estrogen titers derived by sequential hydrolysis (Aliquot B) were still only 76 per cent in the estrone-estradiol fractions and 69 per cent in the estriol fractions of the values obtained by the zinc-hydrochloric acid hydrolysis technique (Aliquot A).

That the marked increase in the estrogen titer obtained by the zinc-hydrochloric acid technique is not peculiar to human pregnancy urine is borne out by the typical hydrolytic data on the urinary estrogen excretion of a man following injection of 2.0 mg. of estrone. The increase of the ratio of estrogen excretion by the two hydrolytic techniques was even greater than that found in human pregnancy urine (Table III).

In these and other studies in which human male urine was used, the urine was divided into aliquots and the hydrolytic technique carried out directly on the urine. Simple rehydrolysis of Aliquot B subsequent to preliminary simple hydrolysis and extraction yielded relatively insignificant estrogen titers, thereby indicating that the time of refluxing does not appear to account for the discrepancy in the estrogen titers obtained by the two hydrolytic techniques.

TABLE III

Effect of Conditions of Hydrolysis on Urinary Estrogen Titer

Male subject, carcinoma of prostate; 2.0 mg. of estrone injected.

Experiment No.	Conditions of hydrolysis	Estrogen titer			
		Estrone	Estradiol	Estrone + estradiol	Estriol
		γ	γ	γ	γ
A	Zinc-HCl; 3 hrs. refluxing	26	107	133	164
B_x	Simple HCl; 20 min. refluxing	14	5	19	52
B_y	" rehydrolysis of B_x; 3 hrs. refluxing	6	0	6	8
	$B_x + B_y$	20	5	25	60
	Ratio, $\frac{A}{B_x + B_y}$			5.3	2.7

Since estriol has not been found in pregnant mare urine, the presence of intermediates which might play a rôle in the conversion of estrone to estriol would seem less likely than in the urine of the pregnant woman. Comparative studies of pregnant mare urine by the two hydrolytic techniques therefore might be expected to throw some light on the problem. Accordingly, simple hydrochloric acid and zinc-hydrochloric acid hydrolysis were carried out on aliquots of a specimen of pregnant mare urine.[1]

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[1] It is recognized that there are present in pregnant mare urine more highly unsaturated estrogens which are not successfully fractionated by our chromatogram.

The data (Table IV) show that there were negligible amounts of unconjugated estrogens in our specimen and that simple hydrolysis yielded slightly higher total estrogen titers than zinc-hydrochloric acid hydrolysis. These data may be interpreted as offering some experimental support to the concept that there are present in the human pregnancy urine intermediate compounds which play a rôle in the conversion of estrone to estriol and which augment the estrogen titer in zinc-hydrochloric acid hydrolysis.[2]

TABLE IV

*Effect of Conditions of Hydrolysis on Urinary Estrogen Titer of Pregnant Mare Urine**

	Chromatographic fraction	Estrogen titer, mg. per gm. powder	
		Kober test	Zimmermann test
No hydrolysis	Estrone	0.014	No test
	Estradiol†	0.006	" "
	Estriol†	0.004	" "
Total		0.024	
Simple hydrolysis, 15 vol. % HCl; 20 min. refluxing	Estrone	0.449	0.450
	Estradiol†	0.123	0.168
	Estriol†	0.051	0.000
Total		0.623	0.618
Zinc-HCl hydrolysis	Estrone	0.003	0.022
	Estradiol‡	0.485	0.000
	Estriol	0.091	0.000
Total		0.579	0.022

* Spray-dried powder reconstituted to approximately normal specific gravity with distilled water.

† Calculated as estrone.

‡ Calculated as estradiol.

A comparison of the effects of the two hydrolytic techniques on essentially estrogen-free urine to which 16-ketoestrone and 16-keto-α-estradiol

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Equilin and equilenin are eluted with estrone and α-estradiol in our technique (unpublished data). However, equilin occurs in relatively small amounts and equilenin does not react to produce the typical Kober color product.

[2] In the absence of more positive proof, we hesitate to attribute the relatively small amounts of material which appeared in our 30 per cent methanol-benzene (estriol) fraction to estriol; the diffuse nature of the trailing boundary of substances eluted principally in a preceding chromatographic fraction would seem a more probable explanation.

have been added would aid in evaluating the capacity of these compounds to augment the estrogen titers by zinc-hydrochloric acid hydrolysis. It must be emphasized that 16-keto-α-estradiol, in our Kober test, gave the typical pink color with about the same density at 520 mμ per microgram as did estrone, whereas pure 16-ketoestrone is practically non-chromogenic (11). 16-Keto-α-estradiol added to pooled male urine after hydrolysis distributed itself between the 5 per cent methanol-benzene and 30 per cent methanol-benzene filtrate fractions in the ratio of approximately 1:1 (Table V). When added to pooled male urine previous to zinc-hydrochloric acid hydrolysis, 16-ketoestrone became chromogenic with the Kober reagent to the extent of 12 per cent, calculated as 16-keto-α-estradiol, and appeared in the 5 per cent methanol-benzene and 30 per cent methanol-benzene filtrate fractions of our chromatogram in the ratio of about 2:1. Similarly, 16-keto-α-estradiol added to pooled male urine previous to zinc-hydrochloric acid hydrolysis was recovered to the extent of 10 per cent in the 5 per cent methanol-benzene fraction and 3 per cent in the 30 per cent methanol-benzene filtrate fraction. Since untreated male urine, upon zinc-hydrochloric acid hydrolysis, did not yield these results, we assume that 16-ketoestrone was reduced to 16-keto-α-estradiol by the zinc-hydrochloric acid hydrolysis and thereby contributed the typical Kober color product. Reduction of 16-ketoestrone

TABLE V

Effect of Simple and Zinc-Hydrochloric Acid Hydrolysis on Titer of Pooled Male Urine

1 liter of male urine with 16-ketoestrone and 16-keto-α-estradiol added.

Experiment No.	Conditions of experiment	Per cent recovery of estrogen in chromatographic fractions		
		2 per cent methanol-benzene (estrone)	5 per cent methanol-benzene (estradiol)	30 per cent methanol-benzene (estriol)
1	1.0 mg. 16-keto-α-estradiol added after simple hydrolysis; 20 min. refluxing	2	40	41
2	2.0 mg. 16-keto-α-estradiol added before Zn-HCl hydrolysis; 3 hrs. refluxing	0	10	3
3	2.0 mg. 16-ketoestrone added before Zn-HCl hydrolysis; 3 hrs. refluxing	0	8	4
4	5.0 mg. 16-ketoestrone added before simple hydrolysis; 20 min. refluxing	0	0	1
	Subsequent Zn-HCl hydrolysis; 3 hrs. refluxing	0.3	1.66	0.58

to 16-keto-α-estradiol with zinc dust and acid is in accord with Huffman and Lott's data (5) on the synthesis of 16-keto-α-estradiol.

Huffman and Lott (5) have commented on the high solubility of 16-ketoestrone in their acid reaction mixture. It occurred to us that the discrepancies in our simple and zinc-hydrochloric acid urine estrogen titers might be explained by the presence of a compound with such high aqueous solubility that it enters the organic phase of our extraction procedure only after partial reduction. Accordingly, 5 mg. of 16-ketoestrone were added to 1 liter of pooled male urine and simple hydrochloric acid hydrolysis and extraction followed by zinc-hydrochloric acid hydrolysis and extraction were carried out sequentially. Although there was some evidence of recovery of estrogens in the second extraction, in percentage figures the results are not very suggestive of the operation of such a mechanism in the case of 16-ketoestrone. In Experiment 4, the 0.3 per cent recovery of 16-ketoestrone as estrone presumably arises from 16-keto-α-estradiol, which, as indicated in Experiment 1, occasionally appears in relatively small amounts in our estrone chromatographic fraction.

The data in Experiments 1, 2, and 3 suggest that 16-ketoestrone, if present in human urine in sufficiently large amounts, could account for some of the additional Kober color products obtained by the Smith zinc-hydrochloric acid hydrolysis technique. Study of the urine of human subjects treated with these compounds, for phenolic estrogens, would present a more favorable test of this concept.[3]

Although these data might seem to offer convincing arguments for the occurrence in human urine of more highly oxygenated estrogens which become chromogenic with the Kober reagent when subjected to zinc-hydrochloric acid hydrolysis, the alternative concept that these results are the consequence of varying relationships between the extent of simultaneous hydrolysis and destruction of the labile estrogens already known to be present in human urine might seem equally valid. This latter concept can be evaluated only if the conjugated forms of the estrogens as they appear in human urine are available for experimentation. Since they are not generally available, only a partial solution to our problem can be attempted at this time. It must be emphasized, however, that current published data (1, 12) on the destructive effects of acid hydrolysis on the conjugated and unconjugated estrogens as derived by bioassay are of little help in evaluating similar phenomena obtained by colorimetric techniques, since the structural features which give rise to a physiological response are not necessarily the same as those which give rise to the colorimetric response. Production of color products with spectral characteristics typical of those obtained with pure compounds is the only criterion of value in estimating recoveries by colorimetric techniques.

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[3] A report on the urinary metabolites of 16-ketoestrone and 16-keto-α-estradiol injected in men will appear in a subsequent publication.

Table VI presents typical data on the recovery of the unconjugated natural estrogens after boiling with strong hydrochloric acid with and without the addition of zinc dust. 20 minutes refluxing with 15 volumes per cent of hydrochloric acid caused not more than 30 per cent destruction of the estrogens, as indicated by the Kober reagent, whereas 3 hours refluxing with 15 volumes per cent of hydrochloric acid and 4 gm. per cent of zinc dust resulted in almost complete destruction of estrogens. This is not surprising, since the amount of zinc dust which was added to the solution (4 gm. per cent) is stoichiometrically equivalent to approximately 10 volumes per cent of hydrochloric acid as used by us, and exerts its action (evolution of hydrogen) actively only during the 1st hour of refluxing. Moreover, the hydrogen ion concentration by glass electrode determination showed no detectable change; *i.e.*, the pH was less than 0.2 over the 3 hour period.

TABLE VI

Recovery of Added Estrogens from Distilled Water and Pooled Male Urine

After boiling for 3 hours with (*a*) 15 volumes per cent of concentrated HCl and (*b*) 15 volumes per cent of concentrated HCl and 4 gm. per cent of zinc dust.

Experiment No.	Solution, 1 liter	Estrogen added, 1.0 mg.	Per cent recovery as shown by Kober test	
			(*a*)	(*b*)
1	H_2O	Estrone	70.4*	0
2	"	α-Estradiol	80.0*	0
3	"	Estriol	92.0*	8
1	Pooled male urine	None	0.0	0
2	" " "	Estrone	73.0	63.0†
3	" " "	α-Estradiol	55.0	86.0
4	" " "	Estriol	38.0	48.0

* 20 minutes boiling substituted for 3 hours.

† Recovered in estradiol fraction of chromatogram.

Typical hydrolytic data obtained by the substitution of acidified pooled human male urine for simple hydrochloric acid solution of the estrogens indicated that the substitution of urine for aqueous solution provided considerable protection to the estrogens against the destructive forces operating in hydrolysis in the presence of zinc dust (Table VI).[4] Estriol appeared to be the most labile of the three estrogens with both hydrolytic techniques. It was observed in experiments in which pooled male urine was used that the stoichiometric excess of hydrochloric acid became an actual excess of zinc, since from 6 to 8 gm. of zinc were recovered routinely at the end of the 3 hour hydrolysis. The titratable acidity at the beginning and the end of hydrolysis changed from an average of 1.5 N to 0.3 N (methyl orange indicator). However, the pH did not rise above 0.2.

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[4] Male urine hydrolyzed by this technique in 1 liter quantities consistently gave estrogen titers within the limits of error reported for our procedure; *i.e.*, ±7 γ of estrone, ±10 γ of estradiol, and ±5 γ of estriol (9).

Data on the destruction of unconjugated estrogens by the hydrolytic process can provide only a partial explanation of estrogen losses during hydrolysis. As Gallagher (13) has emphasized, it is necessary to investigate the water-soluble forms of the phenolic steroids and to devise more reliable methods than are now available for the hydrolytic process based upon the known composition of the conjugates. Some data on this point were provided by the hydrolysis of sodium estrone sulfate added to pooled male urine (Table VII) which show that simple hydrochloric acid hydrolysis yields even greater estrogen recovery than zinc-hydrochloric acid hydrolysis from sodium estrone sulfate. Typical recovery data reported in Table VII represent total estrogen recovered in all three of our chromatographic fractions. In Experiments 1 and 2, less than 10 per cent of the recovered estrogen appeared in the chromatographic estrone and estriol fractions, whereas in Experiments 3 through 9 less than 10 per cent of the recovered estrogen appeared in the estradiol and estriol fractions. The optimum time of refluxing for simple hydrolysis with 15 volumes per cent of hydrochloric acid appeared to be 10 minutes, which yielded 90 per cent

TABLE VII

Recovery of Estrogen from Essentially Estrogen-Free (Pooled Male) Urine

2.0 mg. of sodium estrone sulfate added to 1 liter of urine previous to hydrolysis.

Experiment No.	Conditions of hydrolysis	Recovery by Kober test	Per cent
		mg.	
1	15 vol. % HCl + 4 gm. % zinc; 3 hrs. refluxing	0.563*	40
2	15 " % " + 4 " % " ; 3 " "	0.588*	42
3	15 " % " ; 5 min. refluxing	1.116	80
4	15 " % " ; 10 " "	1.262	90
5	5 " % concentrated H_2SO_4; 10 min. refluxing	1.234	88
6	15 " % HCl; 20 min. refluxing	0.883	63
7	15 " % " ; 30 " "	0.741	53
8	15 " % " ; 40 " "	0.715	51
9	15 " % " ; 60 " "	0.726	52

* Calculated as α-estradiol.

of the theoretical estrone content, whereas the zinc-hydrochloric acid technique of Smith and Smith with 3 hours refluxing gave approximately 40 to 42 per cent recovery. If sulfate is the only form of conjugation for human estrone excretion,[5] then these data offer strong support for the postulate of Smith and Smith; *i.e.*, that conversion of estrone to estradiol and more complete hydrolysis do not account for all of the increased estrogenic potency obtained by the addition of zinc during hydrochloric acid hydrolysis of human female urine.

Unfortunately, sodium estriol glucuronidate was not available to us at the time these studies were made. However, Venning *et al.* (14) have reported quantitative recovery of estriol from sodium estriol glucuronidate added to urine before hydrolysis by their method (approximately 2 volumes per cent of hydrochloric acid and 3 hours autoclaving at 15 pounds pressure). Van Bruggen (12) also has reported quantitative recovery of estriol from sodium estriol glucuronidate in aqueous solution following 10 minutes refluxing in an atmosphere of nitrogen gas with 15 volumes per cent of concentrated hydrochloric acid and 0.125 gm. per cent of protective agent (1-amino-2-naphthol-4-sulfonic acid). Our recovery data of estrone from sodium estrone sulfate together with similar estriol recovery data from sodium estriol glucuronidate, of Van Bruggen, offer strong support for the thesis that sodium estrone sulfate and sodium estriol glucuronidate cannot account for the increased estrogen titers obtained on addition of zinc dust during hydrolysis. The problem therefore resolves itself into a search for (*a*) other conjugate forms of these known natural estrogens or (*b*) discovery of additional estrogen degradation products which are rendered chromogenic with the Kober reagent by the addition of zinc during hydrolysis with hydrochloric acid.

SUMMARY

Comparative colorimetric estimations of estrogens in human pregnancy urine by simple hydrochloric acid and by zinc-hydrochloric acid hydrolysis support the observation of Smith and Smith based on bioassay, that estrone is converted to estradiol by the presence of zinc and that markedly increased estrogen titers are obtained.

Pregnant mare urine does not show augmented titers by the zinc-hydrochloric acid hydrolysis technique.

Recovery of estrone from sodium estrone sulfate added to pooled human male urine prior to zinc-hydrochloric acid hydrolysis was not of high enough order to account for the increased estrogen titers obtained in human pregnancy urine.

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[5] Hydrolytic studies on human pregnancy urine to be reported later seem to indicate otherwise.

16-Ketoestrone added to pooled human male urine reacts to zinc-hydrochloric acid hydrolysis in such a way that its presence in urine could account for some of the augmented Kober color products found by this hydrolytic procedure.

We wish to acknowledge the technical assistance of William M. Conn in this work. We also wish to express our appreciation to Dr. Max Huffman of the Southwestern Medical College, Dallas, Texas, for the 16-ketoestrone and 16-keto-α-estradiol used in this study; to Dr. Herman Cohen of E. R. Squibb and Sons, New Brunswick, New Jersey, for the pregnant mare urine; and to Dr. J. Murray Scott of Ayerst, McKenna and Harrison, Ltd., New York, for the sodium estrone sulfate (synthetic).

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THE OCCURRENCE OF α-ESTRADIOL IN THE URINE OF STALLIONS; ITS IDENTIFICATION AND ISOLATION*

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The estrogenic substances have been demonstrated in many natural tissues and fluids. Because the concentrations in ovaries, in human pregnancy urine, in the urine of pregnant mares, and in the urine of stallions far exceed those found elsewhere, the distribution of the active substances in these four sources has been rather extensively studied. However, even though it has been known since 1934 (1, 2) that stallion urine is very rich in estrogenic material and since 1940 (3) that small amounts of estradiol may be obtained from stallion testes, little or no attention has been given to the non-ketonic estrogens of stallion urine. A thorough search of the literature has revealed no mention of the possible occurrence of non-ketonic estrogens in stallion urine.

For several reasons it seemed desirable to study the distribution of the principal estrogenic constituents of stallion urine, with particular attention to the non-ketonic estrogens, and to this end a systematic investigation of a number of specimens has been made. The results, reported below, show that a considerable and rather well defined proportion of the estrogenic activity of these urines is attributable to α-estradiol and, in fact, pure α-estradiol was isolated as the di-α-naphthoate in good yield from several of the more potent urine specimens. It is also of interest that most such urine specimens contain α-estradiol in far greater concentration than does any other natural source, follicular fluid or ovarian tissue not excluded, hitherto investigated.

Methods

Urine specimens were obtained individually, in so far as was possible, from the various stallions.[1] Care was taken to collect the urines directly

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Preliminary reports of portions of this investigation have been made in a Letter to the Editors (*J. Biol. Chem.*, **158**, 725 (1945)) and before the Thirtieth annual meeting of the Association for the Study of Internal Secretions (*J. Clin. Endocrinol.*, **8**, 701 (1948)).

[1] The author is indebted to Dr. Ralph Reece of the New Jersey Agricultural Experiment Station and to Dr. Robert Bates and Dr. Herman Cohen of E. R. Squibb and

into clean containers and to avoid contamination with fecal matter, etc. The specimens were chilled as soon as delivered to the laboratory (usually within several hours after voiding) and aliquots were hydrolyzed and extracted within 24 to 48 hours.

The hydrolyses, extractions, and purifications were done by well known and accepted methods. For hydrolysis, the urine was acidified to pH 1 with concentrated hydrochloric acid and a small additional excess of the acid was added. The acidified urine was quickly brought to boiling and was refluxed gently for 30 minutes. After rapid cooling under the tap, the hydrolyzed urine was thoroughly extracted by shaking repeatedly with ether. The combined ether extracts were washed with dilute sodium bicarbonate solution until no further color was removed, the combined bicarbonate washings being washed back with a small amount of ether.

The combined ether solutions were distilled to a convenient small volume and were then thoroughly extracted with 1 N NaOH. The combined NaOH solutions were acidified to Congo red with hydrochloric acid and were repeatedly extracted with ether, and the combined ether extracts were again washed with bicarbonate to remove any residual acidic pigment. The ether extract was distilled to dryness and the residual oily material was separated into ketonic and non-ketonic fractions by treatment with Girard's Reagent T in glacial acetic acid solution according to the method of Pincus and Pearlman (4), the quantities of reagents used being increased in proportion to the expected ketone content. The non-ketonic fraction was subjected to at least one further treatment with Girard's Reagent T in order to remove any residual ketonic material which might have escaped the first separation.

The non-ketonic fraction was then fractionated into the so called "weak phenolic" (estradiol) and "strong phenolic" (estriol) fractions by partition between benzene and sodium carbonate solution according to the method of Mather (5) and of Bachman and Pettit (6). After removal of the solvents by distillation, the final products were dissolved in 95 per cent ethyl alcohol, made up to definite volume, and were reserved for estimation of estrogenic content by bioassay and by photofluorometry.

In several cases, in which the estrogenic content of the "weak" nonketonic fraction was sufficiently large to warrant the attempt, the estrogen was further purified with the view of isolating the estrogen, presumed to be α-estradiol. The isolation was attempted in two cases and was successful in each. After completing the desired assays, the remaining material was further purified by repetition of certain of the above pro-

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Sons for their cooperation in obtaining the urine specimens upon which these studies were made.

cedures. The product in each case was then dissolved in benzene and was adsorbed on a column of alumina (Harshaw activated aluminum oxide, No. 350). Fractional elution, with considerable purification, was achieved by washing the column with benzene containing increasing concentrations of ethyl alcohol. Those fractions containing estrogenic activity as indicated by bioassay were combined and the material acetylated with acetic anhydride. The acetyl derivative was sublimed at low pressures (0.03 to 0.04 mm. of Hg), fractions being removed as the temperature was raised. Considerable purification was achieved in this fashion, a semicrystalline product being obtained in each case. The sublimate was saponified and the free estrogen treated with α-naphthoyl chloride as described by MacCorquodale *et al.* (7). The resulting crystalline naphthoate was purified by several recrystallizations from absolute alcohol and from acetone.

The product in each case was identified as α-estradiol di-α-naphthoate by melting points, mixed melting points with authentic α-estradiol di-α-naphthoate, and, in one case, by analysis for carbon and hydrogen.

The ketonic fraction of each urine specimen, derived from the Girard separation, was not further purified. Each was dissolved in 95 per cent ethyl alcohol, diluted to a suitable volume, and aliquots used for the bioassay and photofluorometric estimations described below.

In order to determine the effect of putrefaction on the estrogen partition of stallion urine, aliquots of five specimens were allowed to stand for 2 to 4 months in loosely covered glass containers at room temperature. These "aged" specimens were then worked up as described above and the estrogen titers of the various fractions were compared to those obtained from the fresh specimens from which they were derived.

Bioassays with spayed rats were done by a slight modification of the method of Kahnt and Doisy (8). Similar bioassays were performed with spayed mice, since it has been shown (9) that the ratio of the rat unit to the mouse unit is a good identifying characteristic for the more common natural estrogens.

RESULTS AND DISCUSSION

The total estrogen titers of six fresh specimens of stallion urine are indicated in Table I. It is to be seen that the estrogen titer, as first shown by Zondek (2), is quite inconstant, varying in these specimens from 5500 to 150,000 rat units per liter of urine. The non-ketonic portions of these six specimens were found to contain from 2900 to 140,000 rat units per liter, thus accounting for 45 to 93 per cent of the total estrogenic activity. In five of these six specimens the proportion of the total activity found in the non-ketonic fraction was between 45 and 60 per cent.

By recalculating these data in terms of mg. of estrone and of α-estradiol, it may be seen (Table II) that these six urine specimens contained from 3.3 to 47.4 mg. of estrogen per liter, of which 0.4 to 16.8 mg. per liter (8.0 to 60.4 per cent of the total) was found in the non-ketonic fraction. These figures demonstrate that the α-estradiol titer of these urines was far greater than that of any other tissue or body fluid investigated to date.

The results obtained with the "aged" aliquots of these urines are of considerable interest. These were worked up in the same manner that was described above for the fresh specimens. On assaying the resulting products, it was found (Table III) that in all but one case the total estrogenic activity had decreased considerably during the "aging" period, the losses averaging 33 per cent of the original titer of the fresh aliquots. The assays of the four aged specimens which were partitioned into ketonic and non-ketonic fractions indicate that the loss of activity cannot be ascribed to a consistent reaction. In one case (Preparation 736-743) the loss was apparently due to an extensive and almost stoichiometric

TABLE I

Partition of Estrogens of Stallion Urine

Preparation No.	Stallion	Age	Month collected	Total estrogen	Ketonic		Non-ketonic	
		yrs.		*rat units per l.*	*rat units per l.*	*per cent*	*rat units per l.*	*per cent*
735	Percheron A	?	June	5,500	2,600	47	2,900	53
736	3 pooled	?	"	150,000	10,000	7	140,000	93
747	Percheron A	?	Nov.	71,400	39,600	55	31,800	45
760	?	2	Apr.	20,000	10,400	52	9,600	48
761	Percheron B	10	"	40,000	16,400	40	24,000	60
762	" C	5	"	52,000	27,000	52	25,000	48

TABLE II

Partition of Estrogens of Stallion Urine

Preparation No.	Ketonic as estrone (*A*)	Non-ketonic as α-estradiol (*B*)	Total estrogen (*A* + *B*)	Estrone	Estradiol
	mg. per l.	*mg. per l.*	*mg. per l.*	*per cent*	*per cent*
735	2.9	0.4	3.3	87.9	12.1
736	11.0	16.8	27.8	39.6	60.4
747	43.6	3.8	47.4	92.0	8.0
760	11.4	1.2	12.6	90.5	9.5
761	18.0	2.9	20.9	86.1	13.9
762	29.7	3.0	32.7	90.8	9.2

TABLE III

Estrogen Partition of Fresh and "Aged" Stallion Urine

Preparation No.	Total estrogen			Non-ketonic fraction				Ketonic fraction			
	Fresh	Aged	Change	Fresh	Aged	Change		Fresh	Aged	Change	
	rat units per l.	*rat units per l.*	*per cent*	*rat units per l.*	*rat units per l.*	*rat units per l.*	*mg. per l.**	*rat units per l.*	*rat units per l.*	*rat units per l.*	*mg. per l.†*
735-738	5,500	2,500	−55	2,900				2,600			
736-743	150,000	55,000	−63	140,000	32,000	−108,000	−13.0	10,000	23,000	+13,000	+14.3
760-771	20,000	17,000	−15	9,600	6,300	−3,300	−0.4	10,400	10,700	+300	+0.3
761-772	40,000	23,000	−43	24,000	21,000	−3,000	−0.4	16,400	2,300	−14,100	−15.5
762-773	52,000	58,000	+11	25,000	42,000	+17,000	+2.0	27,000	15,700	−11,300	−12.4

* Non-ketonic estrogen calculated as α-estradiol (1 rat unit $\approx$ 0.12 γ).

† Ketonic estrogen calculated as estrone (1 rat unit $\approx$ 1.1 γ).

conversion of non-ketonic to ketonic estrogen, presumably (see below) by an oxidation of estradiol to estrone. In another (Preparation 760-771) a smaller fraction of the estradiol appeared to have been converted to estrone. In a third specimen (Preparation 761-772), there was a small loss of estradiol and a rather large coincident disappearance of estrone, while in the fourth case (Preparation 762-773) a large proportion of the estrone was lost with a coincident but smaller increase of estradiol, suggesting at least partial conversion of estrone to estradiol.

These figures suggest that in a urine specimen exposed to air both oxidation and reduction of the estrogenic constituents can occur, the extent of the reaction being variable. In some cases, there is an almost stoichiometric conversion of one estrogen to another. In others there is a loss of estrogenically active substances, calculated in terms of mass, and it is probable that in such cases the oxidation-reduction reaction may go so far as to yield non-estrogenic products. Whether such reactions as well as their variability are due to bacterial action or to other mechanisms cannot be definitely stated. It was noted, however, that all these urine specimens became quite alkaline on standing exposed to air, indicating a conversion of nitrogenous substances to ammonia, presumably by bacterial action. In this connection, it is to be recalled that Mamoli (10, 11) demonstrated that many ketonic steroids are readily reduced to the alcoholic analogues by certain microorganisms. It seems equally likely that other organisms may be able to effect the reverse reaction; *e.g.*, oxidation of alcoholic steroids to the corresponding ketones. Indeed, Heller (12) has postulated that such a mechanism operates in surviving liver slices and has provided some evidence to support his hypothesis. It is further not unlikely that such oxidation-reduction reactions may proceed even further with a disruption of the steroid molecule and consequent loss of physiological activity.

Since these mechanisms, bacterial or otherwise, do indeed occur in urine specimens which have been allowed to putrefy, it is understandable that entirely unpredictable changes in composition and titer will be found, depending on the duration of the aging process and upon whether oxidation or reduction of the estrogens is the preponderant reaction. There is no reason to assume that these processes are restricted to stallion urine, and they may be expected to occur as readily in the urine of pregnant mares, human pregnancy urine, etc. Such unpredictable changes, superimposed upon the physiological variations in the urine donors, may explain the disagreement among investigators as to the composition of the "mixed natural estrogens" in such sources as the urine of pregnant mares. It seems necessary to consider the age of the urine specimen, the mode of collection, possibility of contamination, and conditions of aging efore reaching a conclusion as to whether the estrogen partition and titer ound are or are not the same as those of the freshly voided urine.

The ketonic and non-ketonic estrogens were separated as described bove. Estradiol and estriol are the only natural non-ketonic estrogens nown. Any estriol which may have been present should have appeared the "strongly phenolic" non-ketonic fraction derived from the benzene-odium carbonate partition (5, 6). As a matter of fact, in no case was ny activity found in the "strongly phenolic" fractions, thereby indicating hat no estriol was present. Nevertheless it was deemed advisable to haracterize the fractions as accurately as possible as a check on the hemical procedures.

TABLE IV

Characterization of Estrogens of Stallion Urine by Bioassay

Preparation No.	Non-ketonic			Ketonic		
	ml. per rat unit	*ml. per mouse unit*	Rat unit/Mouse unit	*ml. per rat unit*	*ml. per mouse unit*	Rat unit/Mouse unit
729	0.0003	0.0001	3.0			
747	0.0020	0.0006	3.3	0.0016	0.00012	13.3
760	0.0075	0.0012	6.2	0.005	0.0004	12.5
761	0.0021	0.00045	4.7	0.0045	0.00027	16.6
762	0.0019	0.0004	4.7	0.0025	0.00017	14.7
771	0.015	0.0025	6.0	0.0070	0.0005	14.0
772	0.0021	0.00055	3.8	0.030	0.0016	18.7
773	0.0019	0.00045	4.2	0.0055	0.0003	18.3
	γ	γ		γ	γ	
Estrone				1.1	0.08	14.0
α-Estradiol	0.12	0.03	4.0			
Estriol	±1	±24	±0.04			

One means of identifying the natural estrogens is that of comparing the size of the rat unit to that of the mouse unit. As first shown by Westerfeld *et al.* (9), the ratio of the size of the rat unit to that of the mouse unit is quite characteristic for most of the natural estrogens and, in particular, estrone and α-estradiol may be distinguished with ease. This technique was employed on the various fractions obtained, the pertinent results being detailed in Table IV.

As may be seen, the ratios of rat unit to mouse unit were found to be 4:1 and 14:1, respectively, for pure α-estradiol and for estrone. These figures are very near to those previously published by Westerfeld *et al.* (9) and by Levin (13). The ratio for estriol is approximately 0.04:1. It may be seen that the non-ketonic fractions obtained from stallion uri yielded a rat unit to mouse unit ratio varying from 3.0:1 to 6.2:1, mo of the values being between 3.0 and 4.7. Considering the fact that t ratios depend on two bioassays, the figures obtained may be consider to establish that the activity of the non-ketonic fractions was indeed d to the presence of α-estradiol.

The ratios obtained with the ketonic fractions of the same urines we found to be between 12.5:1 and 18.7:1, most falling between 12.5 and 16. These figures, then, are in good agreement with that for pure estro (14:1) and thus indicate that the ketonic fractions owe their activity estrone.

TABLE V

Characterization of Estrogens from Stallion Urine; Bioassay versus Fluorometry

Preparation No.	Non-ketonic			Ketonic		
	Bioassay	Fluoro-metric		Bioassay	Fluoro-metric	
	rat units per ml.	*γ per ml.*	*rat units per γ*	*rat units per ml.*	*γ per ml.*	*rat units p γ*
760	133	13.6	9.8	200	168	1.19
761	476	52.5	9.1	220	261	0.85
762	530	90.0	5.9	400	625	0.64
771	67	13.0	5.2	140	159	0.88
772	480	45.0	10.7	33	42	0.79
773	530	65.0	8.2	182	242	0.75
	rat units per 1 γ			*rat units per 1.1 γ*		
α-Estradiol	8.5		8.5			
Estrone				1		0.91

A further means of characterizing unknown estrogenic substances that of comparing figures for rat units, obtained by bioassay, with thos in terms of mass of estrogenic material, determined by chemical or phys cal methods. The photofluorometric determination of estrogenic steroi as described by Jailer (14) is well adapted for this purpose.

In Table V are given the results obtained by such a comparison of bi assay and photofluorometric data. When estrone and estradiol a treated according to Jailer's method, it is found that they fluoresce equall (14) when compared on a weight basis. It is well established, howeve that estradiol is considerably more active physiologically than is estron Thus in our laboratory 1 γ of estrone is equivalent to 0.9 rat unit, where 1 γ of α-estradiol is equivalent to 8.5 rat units. The ratio of rat units micrograms therefore is 0.9 for estrone and 8.5 for α-estradiol.

The ratios for rat units to micrograms as determined for the no ketonic fractions of stallion urine varied from 5.2 to 10.7 (Table V).[2] Only two of the values were below 8.2. These figures therefore verify the conclusion that the chief estrogenic constituent of these fractions is α-estradiol. Certainly the figures are far too high to permit the deduction that estrone is the major constituent. The two low values may be due to the presence of small quantities of estrone which escaped the repeated treatment with Girard's Reagent T. A more likely assumption, however, is that these values are low due to the presence of non-specific fluorescent material, since these preparations were not pure and since no special attempt was made to use solvents free of fluorescent substances.

The ratios of rat units to micrograms obtained with the ketonic fractions confirm our belief that estrone was the major constituent responsible for the estrogenic activity of these fractions. It may be seen that all the values are very near to that obtained with pure estrone. All but one of the values are slightly lower than the theoretical and, as above, these may also be explained on the basis of the presence in the impure extracts of small amounts of non-estrogenic fluorescent material. The single high value (Preparation 760) is probably due to slight inherent error in the bioassay. It seems improbable that non-ketonic material would be carried over into the ketonic fraction, and certainly there is no reason to assume that this fraction contained a ketonic substance possessing greater physiological activity than estrone.

As described in a previous section, the isolation of the non-ketonic estrogen was attempted in the case of the two most potent preparations derived from stallion urine. The first preparation, L750C, was obtained from the pooled urine of three stallions and is described in Tables I, II, and III as Preparation 736. The second isolation was attempted on Preparation L755, the non-ketonic fraction derived from the pooled urine of an unknown number of stallions.[3] In each of these cases the isolation was successful. The estrogen was isolated as the di-α-naphthoate which after several recrystallizations, yielded analytical data proving the crystals to be pure α-estradiol di-α-naphthoate. The amounts isolated were in fair agreement with the bioassay figures of the preparations before naphthoylation. These data are presented in Table VI.

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[2] The photofluorometric estimations were performed by Dr. J. W. Jailer to whom the author is indebted.

[3] Several years before the present work was begun this preparation was given to the author by a manufacturer of estrogens. It was represented to be a concentrate of pooled stallion urine and to contain large quantities of estrogenic activity in the form of estrone. The ketonic fraction was therefore separated and isolation of the estrone was attempted. Although pure estrone was isolated, the amount obtained was far too small to account for the total activity claimed for the concentrate. After some of the present results on individual specimens were obtained, the non-ketonic fraction of this material, which had been stored in the refrigerator in alcoholic solution, was assayed in this laboratory and was found to contain very large quantities of estrogenic activity. This fraction was therefore worked up, including additional treatment with Girard's reagent, and was found to contain α-estradiol in quantities approximately sufficient to account for the activity as originally reported by the donor of the extract.

It may be seen that the melting points closely approximate those obtained with authentic α-estradiol di-α-naphthoate. On the other hand, in each case there was a definite depression of melting point when mixed with estrone α-naphthoate.

TABLE VI

α-Estradiol Di-α-naphthoate from Stallion Urine; Analytical and Physical Data

Preparation No.			
	Yields		
L750C	200,000 rat units (≈ 24 mg. estradiol) → 20.2 mg. di-α-naphthoate (≈ 9.4 mg. α-estradiol)		
	Recovery 47% (single crop of crystals)		
L755I	150,000 rat units (≈ 18 mg. estradiol) → 36.0 mg. di-α-naphthoate (≈16.8 mg. α-estradiol)		
	Recovery 93% (3 crops of crystals)		
			°C.*
L750C	M. p.		196.5–197.5
	Mixed m.p., L750C with estradiol di-α-naphthoate		197 –198
	" " L750C " estrone α-naphthoate		177 –178.5
L755I	M. p.		193.5–194
	Mixed m.p., L755I with estradiol di-α-naphthoate		195 –196.5
	" " L755I " estrone α-naphthoate		184 –203
		C	H
	Elementary analysis	*per cent*	*per cent*
L750C	Found	82.75	6.26
	Calculated. $C_{40}H_{36}O_4$ (estradiol di-α-naphthoate)	82.72	6.25
	" $C_{29}H_{28}O_3$ (estrone α-naphthoate)	82.04	6.65

* All melting points uncorrected.

Elementary analysis was performed[4] on one of these preparations, L750C, and the analytical figures (Table VI) correspond very closely to the theoretical for α-estradiol di-α-naphthoate.

It is therefore definite that the non-ketonic estrogen, accounting for 45 to 93 per cent of the total estrogenic activity of stallion urine, is indeed α-estradiol. This is established by the chemical manipulations and partitions used in the separation of these fractions from other estrogenic fractions, by the ratio of size of rat to mouse unit, by the ratio of rat unit to weight of estrogen as determined by photofluorometric methods, and, finally, by the isolation of the estrogen as the di-α-naphthoate and determination of its purity by melting point, mixed melting point, and elementary analysis.

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[4] The elementary analysis was made by Dr. W. Saschek of the Department of Biochemistry of this institution.

The fact that these high titers of α-estradiol have been observed in fresh urine specimens and that losses of this estrogen with coincident but non-proportional changes in estrone titer were observed in specimens allowed to age under non-sterile conditions indicates that considerable care must be exercised in making statements concerning the relative amounts of the natural estrogens of equine urines unless the freshness, manner of aging, and degree of deterioration are known and specified.

The author wishes to express his appreciation to Miss Barbara Singer whose assistance with the many bioassays greatly facilitated the progress of this investigation.

SUMMARY

The estrogenic substances of a number of specimens of stallion urine, immediately after collection and after several months of aging, have been partitioned into ketonic, "strongly acidic" non-ketonic, and "weakly acidic" non-ketonic fractions. The "strongly acidic" non-ketonic (*i.e.*, estriol) fraction was inactive. The "weakly acidic" non-ketonic (estradiol) fractions of freshly voided urines accounted for 45 to 93 per cent of the total estrogenic activity.

The ketonic estrogen has been identified as estrone by means of the mode of separation of the ketonic fraction, by comparison of ratio of size of rat unit to mouse unit, and by comparison of physiological activity to the mass of estrogenic material as determined by photofluorometric analysis.

By the same methods, the "weakly acidic" non-ketonic estrogens have been identified as α-estradiol. In addition, the non-ketonic estrogen of several specimens has been naphthoylated and the isolated derivatives proved to be α-estradiol di-α-naphthoate by melting points, mixed melting points, and elementary analysis.

The data therefore verify the finding that fresh stallion urine contains large quantities of estrogenic substances and establishes that a large proportion of this estrogen is α-estradiol. Allowing the urine to undergo putrefaction causes a variable but appreciable loss of estradiol with coincident variable, but not necessarily proportional, changes in the estrone content. Possible reasons for these changes are discussed.

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MALONATE INHIBITION OF OXIDATIONS IN THE KREBS TRICARBOXYLIC ACID CYCLE*

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The inhibition of succinate oxidation by malonate is a well known phenomenon. Since the oxidation of succinate to fumarate is an integral part of the Krebs cycle of oxidations, it has been generally assumed that the inhibitory effect of malonate upon the oxidation of any member of the cycle is the result of the inhibition of the succinate to fumarate step. However, the present paper provides evidence that malonate inhibits oxidations in the cycle by at least two mechanisms: in addition to the inhibition resulting from a block of succinate oxidation, malonate inhibits oxidation by another mechanism that is believed to involve combination with magnesium ions.

Methods

The reaction mixture has been described elsewhere (1). It contained (final concentrations) M/15 KCl, M/60 K phosphate at pH 7.2, 1.3×10^{-5} M cytochrome *c*, and 0.001 M K adenosine triphosphate. The concentrations of $MgCl_2$, oxalacetate, pyruvate, K malonate, and rat tissue homogenate are given for the separate experiments. When oxygen uptake measurements were desired, the Warburg apparatus was used in the conventional manner at 38°. Samples were equilibrated for 10 minutes and then readings were taken at 10 minute intervals. For some experiments substrate disappearance was considered a better measure of the reaction than oxygen uptake. When the rate of oxygen uptake was not required, samples were shaken in open flasks. At the end of the experiment 2 ml. of 17.5 per cent trichloroacetic acid were added to give a final concentration of 7 per cent; the precipitated protein was centrifuged and the supernatant fluid analyzed. All experiments were in duplicate, and the more important ones were carried out on two or more animals. The rats were all young adult males on a stock diet.

Total keto acids were determined colorimetrically with 2,4-dinitro-

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phenylhydrazine (2). In some cases readings of light transmission were taken at both 480 and 580 mμ in order to determine α-ketoglutarate in the presence of oxalacetate and pyruvate (2). Since the amount of α-ketoglutarate found was relatively small, all of the keto acid was calculated as oxalacetate, except in the experiments with variable magnesium, in which the α-ketoglutarate was subtracted from total keto acids and the difference was called oxalacetate. This figure is really the sum of oxalacetate plus pyruvate, since it is not possible to distinguish between these compounds by the method as used. For the purposes of these experiments this fact is advantageous, since conversion of oxalacetate to pyruvate does not result in keto acid disappearance. Citrate was determined by the method of Natelson, Lugovoy, and Pincus (3).[1]

Results

The inhibition observed when malonate is added to the reaction mixture used for the study of oxalacetate oxidation depends not only upon the substrates employed but upon the kind of tissue used for the homogenate. Table I summarizes the results obtained with heart, kidney, brain, and liver homogenates, without substrate, and with oxalacetate, pyruvate, and a mixture of oxalacetate and pyruvate. Each substrate combination was tested without malonate and with malonate at final concentrations of 0.004 and 0.02 M. The lower concentration provided an effective block for the succinate to fumarate step under the conditions of these experiments, in which diffusion barriers were less effective than in slices, and in which no succinate was added. That the higher malonate concentration inhibited not only the succinate to fumarate step but also the conversion of oxalacetate and pyruvate to the tricarboxylic acids is indicated by the data to follow. The experiments also provide additional data regarding the alternative metabolic pathways in the various tissues.

"Low" Malonate—Table I shows that liver differs from the other tissues with respect to its ability to metabolize pyruvate. This is shown by the rates of oxidation with pyruvate as the sole substrate with the low level of malonate. In the case of heart and kidney, the control and inhibited rates under these conditions were 89 and 8 and 74 and 5 microliters of oxygen per 20 minutes respectively, while with liver the rates were 68 and 49 microliters. Brain resembled heart and kidney but the effect of malonate was less definitive. The result with liver is in agreement with Lehninger's finding (4) that washed residues from isotonic liver homogenates can oxidize pyruvate to acetoacetate in the presence of malonate. Since heart and kidney homogenates could not burn pyruvate in the pres-

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[1] We wish to thank Mrs. G. G. Lyle for carrying out this determination.

nce of malonate, these tissues must have very little of the alternative athway possessed by liver. Apparently they are also unable to convert yruvate to acetate to any appreciable extent, at least in the absence of xalacetate.

The inhibition of pyruvate oxidation by 0.004 M malonate in kidney and heart was interpreted in terms of an inhibition of the succinate to fumarate step. Support for this interpretation was furnished by the experiments with oxalacetate alone, which is converted to a mixture of oxalacetate and pyruvate, as well as by experiments with both substrates. (The mixture was more rapidly oxidized in brain and in heart but not in liver or kidney homogenates (*cf.* (1).) In brain and heart the oxalacetate decarboxylase must have been the rate-limiting factor when oxalacetate was the sole substrate (see Table I).) Whether oxalacetate alone or the mixture was used, the inhibition by the low level of malonate was very much less than that observed when pyruvate was the sole substrate in kidney and in heart homogenates. By preventing the *formation* of oxalacetate in the system

TABLE I

Effect of Malonate on Oxygen Uptake

Conditions as in the text plus 0.003 M $MgCl_2$, 6.7×10^{-3} M K phosphate (pH 7.2), and 0.0035 M pyruvate, oxalacetate, or an equimolar mixture. The wet weight of each tissue is given in the table and the tissues were used as 10 per cent homogenates in isotonic KCl. The values are in microliters of O_2 taken up between 10 and 30 minutes after the flasks were put on the Warburg apparatus. The results are averages of experiments with homogenates from two different animals.

Tissue	Substrate	Malonate		
		0.0 M	0.004 M	0.02 M
Heart, 40 mg.	None	3	2	2
	Pyruvate	89	8	6
	Oxalacetate	97	67	56
	Mixture	196	87	45
Kidney, 30 mg.	None	6	4	1
	Pyruvate	74	5	4
	Oxalacetate	138	93	42
	Mixture	146	66	23
Brain, 50 mg.	None	15	6	4
	Pyruvate	46	12	12
	Oxalacetate	57	29	18
	Mixture	74	39	19
Liver, 50 mg.	None	27	15	13
	Pyruvate	68	49	38
	Oxalacetate	86	71	40
	Mixture	85	72	42

containing pyruvate alone, the low level of malonate could inhibit pyr vate oxidation via the Krebs cycle, but when oxalacetate was added, th importance of the succinate to fumarate step was minimized, and th effect of malonate decreased. However, even when oxalacetate wa added, the inhibition was not overcome and this raised the question whether the malonate had a direct effect on oxalacetate oxidation whether the effect was still due to the blocked succinate oxidation.

In the case of oxalacetate plus malonate, a block in the cycle will sti have a strong effect on the oxidation rate, because the amount of adde oxalacetate is necessarily fairly low (1), and unless it is replenished by th functioning of the cycle, the pyruvate cannot be oxidized via the cycl On the other hand, larger amounts of fumarate can be added, to give continually renewed supply of oxalacetate, in which case the experiment with heart and kidney homogenates are comparable to those by Krebs an Eggleston with minced pigeon breast muscle (5, 6). With the usua amounts of homogenate and periods of incubation, a plot of oxygen uptak against fumarate concentration revealed a constant inhibition of betwee 10 and 20 per cent by 0.004 M malonate over a fairly wide range of fumarate concentrations, when pyruvate was constant at 0.0053 M and oxalacetate was omitted. The result obtained with 0.0053 M fumarate may be seen in Fig. 1 (among other data, see below). This amount of inhibition was less than the theoretical 20 per cent for a cycle with the succinate to fumarate step specifically inhibited. This small inhibition may be explained partly on the basis that the latter step was probably not contributing the theoretical fraction to the control rate, and partly on the basis of the effect of malonate described below.

"High" Malonate—The results cited above suggest that the main effect of malonate at the low level of 0.004 M is to block the Krebs cycle at the succinate-fumarate step. Furthermore, the data suggest that for this fraction *the block is essentially complete* at this level of malonate. But the data in Table I show that when the malonate concentration was increased from 0.004 to 0.02 M a *further marked reduction* in the rate of oxidation of oxalacetate plus pyruvate was observed. If the succinate step is blocked by the low level of malonate, the effect produced by higher levels must be elsewhere. In Fig. 1 is presented the effect of malonate on various substrate combinations. The system containing fumarate and pyruvate is the one that is least dependent upon the succinate step, and it was affected but little by 0.004 M malonate, as mentioned earlier. However, as the concentration of malonate was increased to 0.02 M, a marked inhibition was observed with the fumarate-pyruvate combination, and parallel results were obtained with oxalacetate alone. The curves in Fig. 1 fall into two categories, with the pyruvate alone and the succinate showing strong inhibition by 0.004 M malonate and the oxalacetate alone,

and pyruvate plus fumarate showing less inhibition at 0.004 M malonate but rather a progressively increasing inhibition up to 0.02 M malonate. Although these data suggest that the higher malonate level had a direct effect upon the oxalacetate oxidation *per se*, analytical data were needed to supplement the measurements of oxygen uptake.

Analytical Data—If malonate could inhibit oxalacetate oxidation directly, one might expect to obtain a decrease in oxalacetate disappearance in the presence of malonate. Since malonate has been reported to inhibit the decarboxylation of oxalacetate (7), the experiments were performed with a mixture of oxalacetate and pyruvate. The results are shown in Fig. 2. The effect on keto acid disappearance is closely correlated with the effect on oxygen uptake described in Fig. 1. The oxidation of oxalacetate requires magnesium ions, and the inhibition by malonate depends upon the concentration of magnesium, as shown in Fig. 3. The theoretical aspects of these curves will be discussed later. Here it may be stated that neither succinate nor malate oxidation *per se* requires magnesium ions.

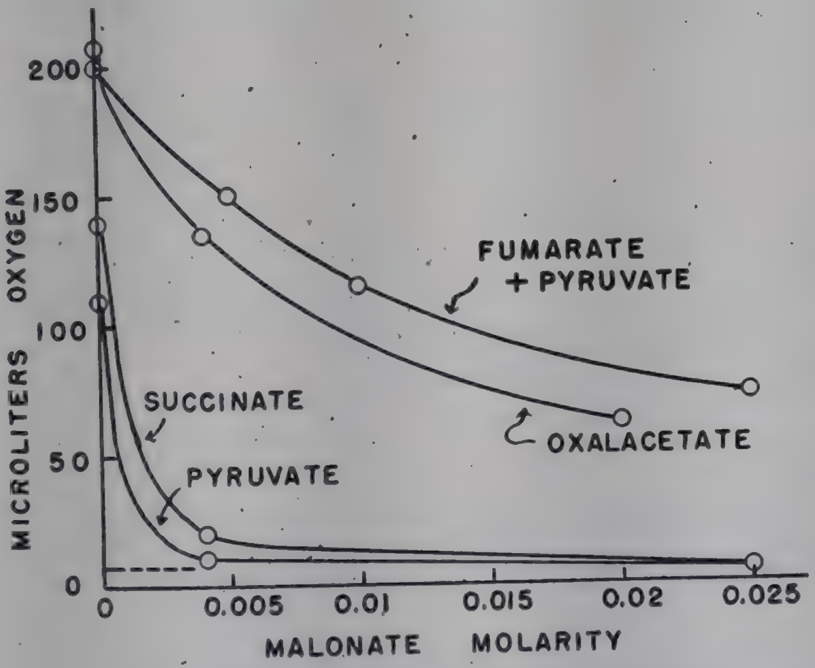

FIG. 1. Effect of malonate concentration on the oxidation of various intermediates in the Krebs tricarboxylic acid cycle. The data are given in microliters of oxygen in 30 minutes following a 10 minute equilibration. Conditions as in Table I with 0.3 ml. of 10 per cent isotonic KCl kidney homogenate per flask. Substrate concentrations, fumarate plus pyruvate, each at 0.0053 M; oxalacetate, 0.0035 M; succinate, 0.0033 M; pyruvate, 0.0035 M.

The inhibition of oxalacetate disappearance does not necessarily indicate a direct effect by malonate. However, measurements of citrate accumulation might be expected to be a more direct indication of a bloc near the Krebs condensation. Table II shows that malonate *decrease* citrate accumulation, and in addition shows that very little citrate accumulates when pyruvate is the substrate. A block at the succinate ste would not be expected to decrease citrate formation unless the supply o oxalacetate became limiting, which is unlikely under these conditions.

To localize further the block produced by the higher levels of malonate experiments were carried out on the single step, malate to oxalacetate When malate is oxidized to oxalacetate, the reverse reaction is so stron

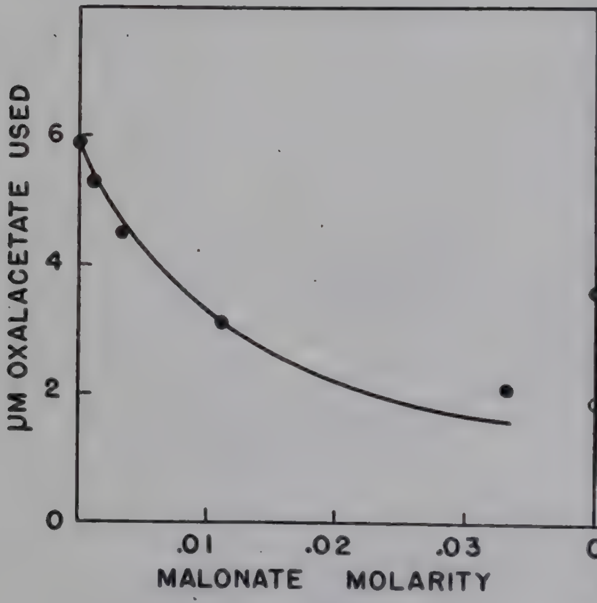

FIG. 2

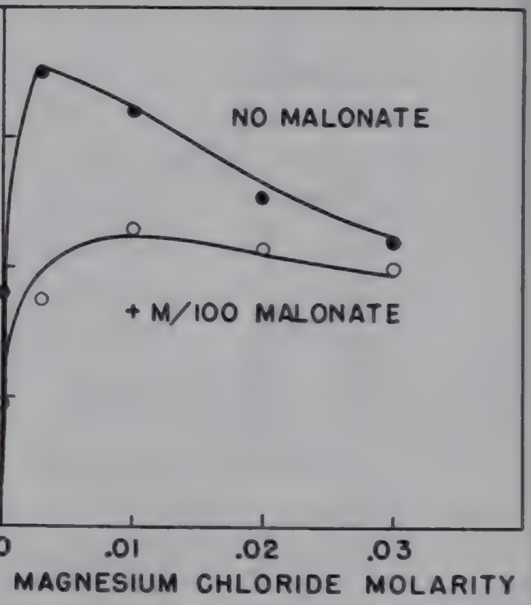

FIG. 3

FIG. 2. Experimental points and theoretical curve of oxalacetate disappearance in 20 minutes *versus* final malonate concentration. Conditions as in the text plus 0.0033 M $MgCl_2$, 0.003 M oxalacetate, 0.003 M pyruvate, and 0.3 ml. of 10 per cent water homogenate of rat kidney.

FIG. 3. Experimental points and theoretical curves of oxalacetate disappearance in 25 minutes *versus* final $MgCl_2$ concentration. Conditions as in the text plus 0.003 M oxalacetate and 0.3 ml. of 10 per cent water homogenate of rat kidney.

that, unless the oxalacetate is removed, the oxidation stops (8). Table III shows that malonate strongly inhibited malate oxidation, but, at the same time, the rate of keto acid accumulation was *increased*. This fact alone would suggest that the malonate blocked keto acid removal rathe than malate oxidation, but further support for this interpretation is gaine from the fact that 0.01 M malonate does not inhibit malate oxidation in a system in which oxalacetate is removed by transamination (8). If the accumulation of keto acid in this experiment were to be ascribed to a succession of back-reactions from the succinate block, one would expect an increase in citrate, but here a *decrease* was found (Table II).

Attempts were made to learn whether malonate acted as a competitive inhibitor for oxalacetate, pyruvate, or a pyruvate derivative. Since both oxalacetate and malonate inhibit a succinic enzyme, one might expect malonate to inhibit an oxalacetic enzyme. However, no support for this idea was obtained, since increases in pyruvate had no effect and increases

TABLE II

Citrate Production

Liver, sample at 20 minutes, conditions as in the text plus 0.0053 M substrate, 0.4 ml. of 10 per cent liver homogenate in isotonic KCl. Brain, sample at 70 minutes, conditions as in the text plus 0.003 M MgCl$_2$, 0.0045 M substrate, 0.8 ml. of 10 per cent rat brain homogenate in isotonic KCl.

The results are given in micromoles of citrate per flask.

Additions	Liver	
	No malonate	0.03 M malonate
Oxalacetate, 0.003 M MgCl$_2$	1.65	0.81
" 0.036 " "	0.93	0.70
Pyruvate, 0.036 M MgCl$_2$	0.07	0.04
	Brain	
	No malonate	0.01 M malonate
Oxalacetate	0.70	0.50
1 oxalacetate to 4 pyruvate	0.41	0.28
Pyruvate	0.07	0.06

TABLE III

Oxygen Uptake and Production of Keto Acid from Malate

Conditions as in the text plus 0.003 M MgCl$_2$, 0.0027 M malate, and 0.3 ml. of 10 per cent rat kidney homogenate in isotonic KCl. The data are in micromoles of keto acid found per flask at 0 to 40 minutes, and microliters of O$_2$ taken up in the 10 minutes following equilibration.

Time	Malonate		
	0.0 M	0.01 M	0.03 M
min.			
0	0.03	0.04	0.02
10	0.10	0.19	0.16
20	0.13	0.20	0.19
40	0.17	0.21	0.21
O$_2$, 10–20 min.	23	5	0

in oxalacetate actually increased the inhibition. The latter effect may have involved the magnesium ion in a manner analogous to the effect of malonate, which will be discussed below.

DISCUSSION

The above data show that the oxidation of oxalacetate to tricarboxylic acid is inhibited by malonate, and that Mg^{++} decreases this inhibition, though high concentrations of Mg^{++} also inhibit.

Malonate could cause the observed effect by at least two possible mechanisms. It might act as a competitive inhibitor by combining with the enzyme at a location where Mg^{++} must combine. This seems unlikely because Mg^{++} and malonate have opposite charges and are structurally dissimilar. A second possibility is that malonate forms a complex with Mg^{++}, lowering the effective concentration of Mg^{++} in the solution. A binary complex with a dissociation constant of 10^{-2} has been reported (9).

The question now arises as to whether this constant is of the right magnitude to explain the observations. An equation in which v is the

$$v = \frac{VK_1\text{Mg}}{(1 + (1 + 100\,\text{M})K_1\text{Mg})(1 + K_2\text{Mg})} \tag{1}$$

velocity of the reaction, and Mg and M are the concentrations of dissociated Mg^{++} and dissociated malonate, was used to plot the curves in Figs. 2 and 3. The constants V, K_1, and K_2 were determined from three experimental points in Fig. 3, with malonate absent, and had the values 9.0, 1.9×10^3, and 32, respectively. The constant V depends on the amount of enzyme present and was determined separately for Fig. 2 from the value obtained with no malonate present. The equation was derived by assuming that Mg^{++} reversibly combines with enzyme as a cofactor with association constant K_1 and as an inhibitor with association constant K_2, and that malonate inhibits by forming a complex with Mg^{++} combined as a cofactor, as well as by lowering the concentration of free Mg^{++}. This would be similar to the inhibition of enolase by fluoride, in which an inactive fluorophosphate is formed with Mg^{++} bound to the enzyme (10). We conclude that complex formation could account for the inhibition by malonate, because the data obtained with malonate fit the equation derived from data in the absence of malonate.

When malonate is added to a reaction mixture, it should not be assumed that its only effect is to prevent oxidation of succinate. It can also inhibit the oxidation of oxalacetate, and since a number of other enzyme systems also require Mg^{++} as a cofactor the effect on these systems should also be taken into account. It should also be noted that addition of excess Mg^{++} does not bring the rate of reaction back to its maximum value.

In attempting to block the conversion of succinate to fumarate in the cyclic oxidation of oxalacetate, malonate cannot be used without affecting oxalacetate removal. It would appear from Fig. 1 that the greatest differentiation between the two steps can be obtained with a malonate concentration of about 0.004 M, although it is clear that this has some effect on the oxalacetate step, while it is not completely effective in blocking the succinate step. Moreover the blocking of succinate oxidation with this level of malonate is a function of the succinate concentration. From the Michaelis-Menten equation in the form

$$v = \frac{VS}{K_s\left(1 + \frac{I}{K_i}\right) + S} \tag{2}$$

$$\frac{v_i}{v_0} = \frac{K_s + S}{K_s\left(1 + \frac{I}{K_i}\right) + S} \tag{3}$$

in which v_i is the velocity in the presence of malonate at molarity I and succinate at molarity S, v_0 is the velocity with no malonate but with substrate at molarity S, and K_s and K_i are the dissociation constants of succinate and malonate respectively. From this equation, using 6×10^{-3} M for K_s, 1×10^{-4} M for K_i (11), and 0.004 M for I, one obtains a theoretical value of 0.037 for v_i/v_0 when S is 0.0033 M. When the value for I is corrected for the effect of the Mg (see above), the value for v_i/v_0 becomes 0.046. In Fig. I, the observed value for v_i/v_0 at 0.0033 M succinate was 0.11 when corrected for the endogenous respiration and 0.15 with no correction. The agreement with the theoretical figure is fairly good, although the observed inhibition was lower than the theoretical. It seems likely that the actual extent of the inhibition of the succinate step by malonate under the conditions described for Fig. 1 is closer to the theoretical on the following basis: The data for both v_i and v_0 are based on a 30 minute period, during which both rates were declining. However, in the case of the v_0 rate the decline was largely due to the rapid decrease in S, and should be extrapolated to zero time to get the true value of v_0 when S is 0.0033 M. On the other hand, the rate for v_i could not have declined because of a decrease in S, since the rate was so low that the decrease in S was small in comparison with the original amount. The original rate in the case of the v_i measurement included a larger non-succinate component than the v_0 measurement, but at this level of respiration the adenosine triphosphate would not be maintained and the rate would decline as the system reverted to succinate oxidation. Thus the final rate in the v_i measuremen might be more logically compared with the initial rate from the v_0 measurement. Taking the rate at 30 minutes as the final v_i rate, we obtained a value of 0.066 for v_i/v_0. Thus the theoretical inhibition was 95.4 per cent, and the observed value was 89 per cent uncorrected and 93.4 per cent when corrected. From Equation 3, the theoretical inhibition as succinate approaches zero is 97 per cent at 0.004 M.

Thus the succinate step can be fairly well blocked with 0.004 M malonate in the oxalacetate-oxidizing system *when no succinate is added.* If succinate is added or if the experiment is continued long enough to accumulate appreciable amounts of succinate, the effectiveness of the block will decrease to an extent that can be calculated with the aid of Equation 3 and the dissociation constant for magnesium malonate. However, it would appear that it will not be possible to inhibit succinate oxidation effectively without partially inhibiting oxalacetate oxidation.

SUMMARY

1. Malonate inhibits oxidations in the Krebs tricarboxylic acid cycle in fortified homogenates by at least two mechanisms.

2. In addition to the well known inhibition of succinate oxidation, malonate inhibits the oxidation of oxalacetate.

3. The inhibition of oxalacetate oxidation by malonate was shown to depend on the concentration of magnesium ions, and it was shown that the effect could be explained in terms of the formation of a complex of malonate with free and bound magnesium.

4. Observations on various tissues and substrate combinations were discussed in terms of the Krebs tricarboxylic acid cycle.

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THE INFLUENCE OF TEMPERATURE AND HYDROSTATIC PRESSURE ON THE DENATURATION OF METHEMOGLOBIN BY URETHANES AND SALICYLATE*

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The denaturation of methemoglobin by sodium salicylate was found by Anson and Mirsky (1) to be reversible upon removal or dilution of the salicylate and to have essentially no temperature coefficient. Evidence from kinetic studies has shown (2–7) that several proteins are denatured by heat and by urethane (reversibly or irreversibly, depending on the conditions and the protein) with a high temperature coefficient and with a volume change of the order of +50 to +100 cc. per mole. Because of the volume increase these denaturations are opposable by pressure (8).

Methemoglobin provides a protein system favorable for spectroscopic and colorimetric study, since, in solutions of 1 per cent or less, both the native form and the reversibly denatured product in the presence of salicylate are known to be soluble, and because a marked and characteristic difference in the visible light absorption spectrum exists between the two forms. This paper reports a study of the effects of pressure, temperature, sodium salicylate, and urethanes on the methemoglobin system.

EXPERIMENTAL

The colorimeter and the pressure-resistant absorption cell, referred to as the bomb, were described previously (9). Advantage is taken of the difference between native and denatured methemoglobin in the absorpton of the energy of the 546 mμ mercury line for the quantitative measure of the two forms.

In some experiments, the methemoglobin and denaturant were mixed under pressure, by using specially made, very thin walled glass vials into which could be sealed the material to be introduced into the solution in the bomb after pressure was applied. Pressures up to 680 atmospheres can be applied quickly, and as a result the glass vial is crushed.

For making spectrograms of solutions under pressure a special carriage for the colorimeter bombs was made to replace the standard cell carriage of the model DU Beckman spectrophotometer. Because the glass windows are about 2 cm. thick, with openings in the bomb of only 0.5 cm. in diam-

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eter, and because the light path in the solution in the bomb is 5 cm. lon instead of the standard 1 cm., optical density readings of the Beckma instrument, taken while the bombs are being used as cells, cannot be quantitatively compared to readings made with the standard absorption cell However, comparisons between spectrograms of the same solution, unde normal and increased pressure, respectively, are valid, as well as comparisons of the contours of the other spectrograms in general.

Methemoglobin solutions were made from bovine blood and stored according to the methods described by Anson and Mirsky (10). The lake blood was filtered through a Berkefeld filter in order to remove the stroma before being used or stored. Solutions of methemoglobin in a final concentration of 0.1 per cent were employed in all experiments except in the spectroscopic studies of solutions under pressure, in which 0.05 per cent solutions were used.

Reversal of methemoglobin denaturation upon dilution of the salicylate and the independence of temperature of this native denatured methemoglobin equilibrium were confirmed.

Methemoglobin in solutions of urethane of concentrations greater than 4.5 M have the same red color as methemoglobin in 0.5 M sodium salicylate. This salicylate concentration, according to Anson and Mirsky (10), gives complete denaturation. Fig. 1 shows the correspondence between the spectrograms of methemoglobin in 4.5 M urethane and methemoglobin in 0.5 M salicylate, in contrast to that of native methemoglobin. Dilution or removal of salicylate to 0.2 M results in a solution whose spectrogram is shown in Fig. 1, Curve 3, indicating a partial reversal of the reaction. Dilution of the urethane solutions, however, results in precipitation of the protein and, although the red color disappears, the reaction is not similarly reversed.

At 24° methemoglobin is precipitated from solutions in proportion to the urethane concentration, in the range of 1 to 2.5 M. In concentrations above 2.5 M the amount of precipitate decreases and the supernatant liquid becomes increasingly red in color until, when methemoglobin is added to a urethane solution to make it 4.5 M in urethane and 0.1 per cent methemoglobin, there is no visible precipitation. In this case, the spectrogram of Fig. 1, Curve 2, is obtained. The spectrogram of the supernatant liquid from a 2 M urethane solution is represented by Curve 5 of Fig. 1. The spectrogram of methemoglobin in 0.5 M urethane is indistinguishable from that of native methemoglobin.

All methemoglobin solutions in concentrations of urethane greater than 1 M respond to temperature change. If precipitation at 24° is incomplete, an increase in temperature will effect essentially complete precipitation somewhat before the temperature reaches 60°, or at lower temperatures f the concentration is increased from 1 to 2.5 M. The red solution characteristic of the liquid phase in the urethane concentrations greater than 2.5 M becomes straw-colored above 55°. This color is to the naked eye indistinguishable from that of native methemoglobin, but it does not have the same spectrogram, as shown in Fig. 2. The color is completely reversed on cooling to the former red color as shown by Curve 4 of Fig. 2.

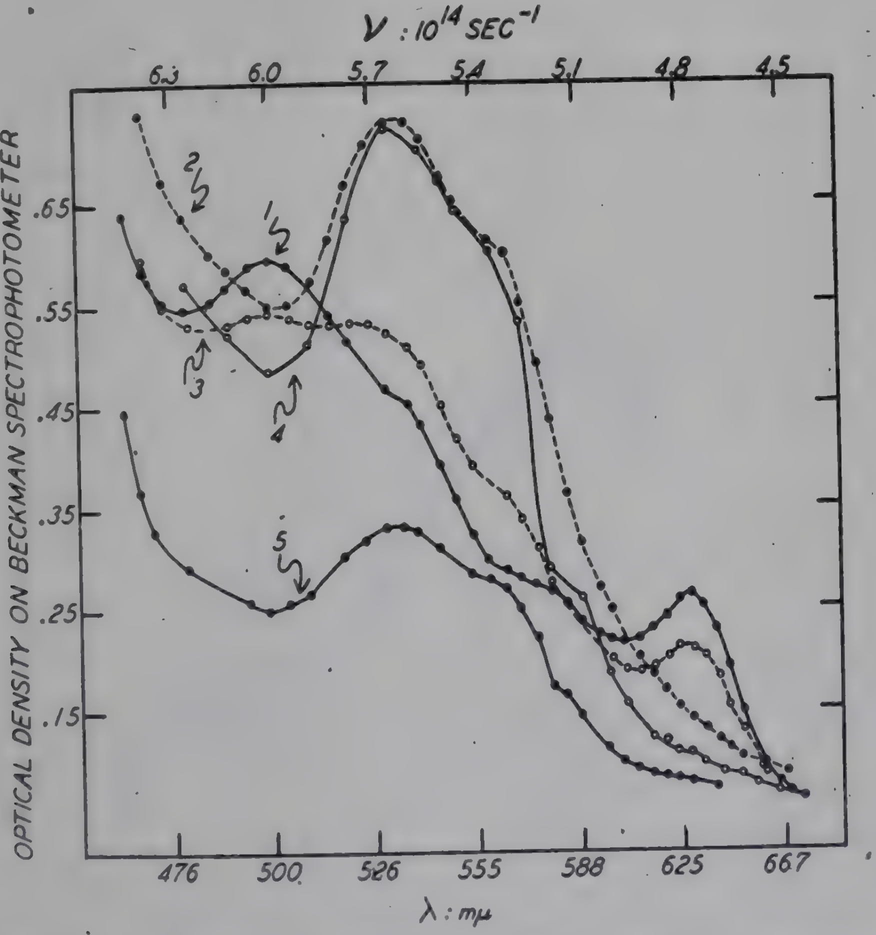

FIG. 1. Curve 1, 0.1 per cent native methemoglobin; Curve 2, 0.1 per cent methemoglobin in 4.5 M urethane; Curve 3, 0.1 per cent methemoglobin in 0.2 M sodium salicylate; Curve 4, 0.1 per cent methemoglobin in 0.5 M sodium salicylate (complete denaturation); Curve 5, supernatant liquid from the precipitate of a solution made up to 0.1 per cent methemoglobin and 2 M urethane.

A solution of native methemoglobin at 60° during a short exposure to this temperature has the same spectrogram as at 24°.

The application of pressures up to 680 atmospheres had no detectable effect on the equilibrium between native and denatured methemoglobin in the presence of salicylate in concentrations between 0.1 and 0.5 M, the range in which denaturation varies from 0 to 100 per cent according to Anson and Mirsky (1). Likewise pressure had no effect on the light absorption by methemoglobin in 4.5 M urethane. After crystalline salicylat had been added under 680 atmospheres of pressure to a methemoglobi solution in the colorimeter bomb, a sudden increase of 20 per cent in ab sorption occurred if the pressure fell below about 6 atmospheres. Appl cation of pressure again did not change the absorption, nor did the releas of it a second time cause further increase of absorption. The spectrogram

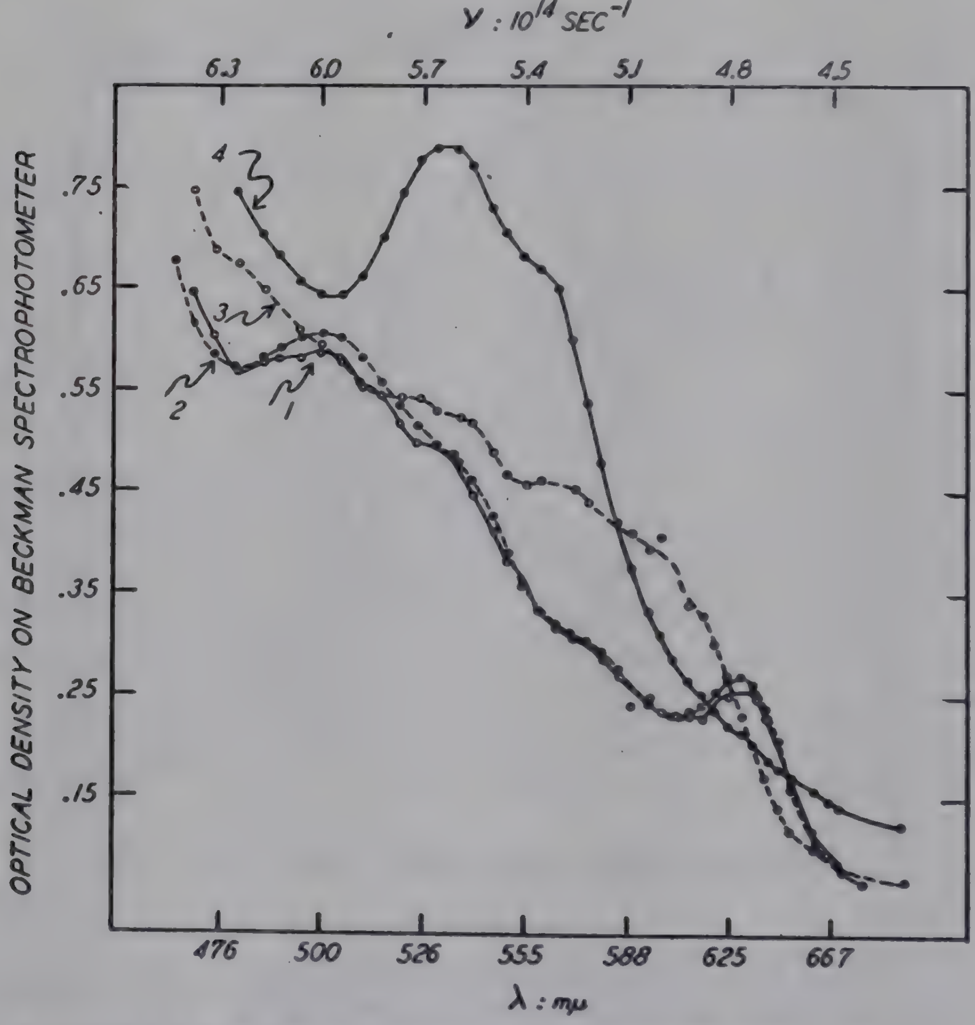

FIG. 2. Curve 1, 0.1 per cent native methemoglobin at 60°; Curve 2, solution coole to 24° after spectrogram of Curve 1 was taken; Curve 3, 0.1 per cent methemoglobi in 4.5 M urethane at 60°; Curve 4, solution cooled to 24° after spectrogram of Curve was taken.

before and after pressure release on a methemoglobin solution to whic crystalline sodium salicylate was added under 680 atmospheres of pressur are shown in Fig. 3. The spectrogram, under pressure, of a sodium sali- cylate solution to which methemoglobin was added under pressure is als shown in Fig. 3, Curve 3.

A series of saturated solutions of various urethanes in methemoglobi was prepared. The urethane used and the temperature at which tur-

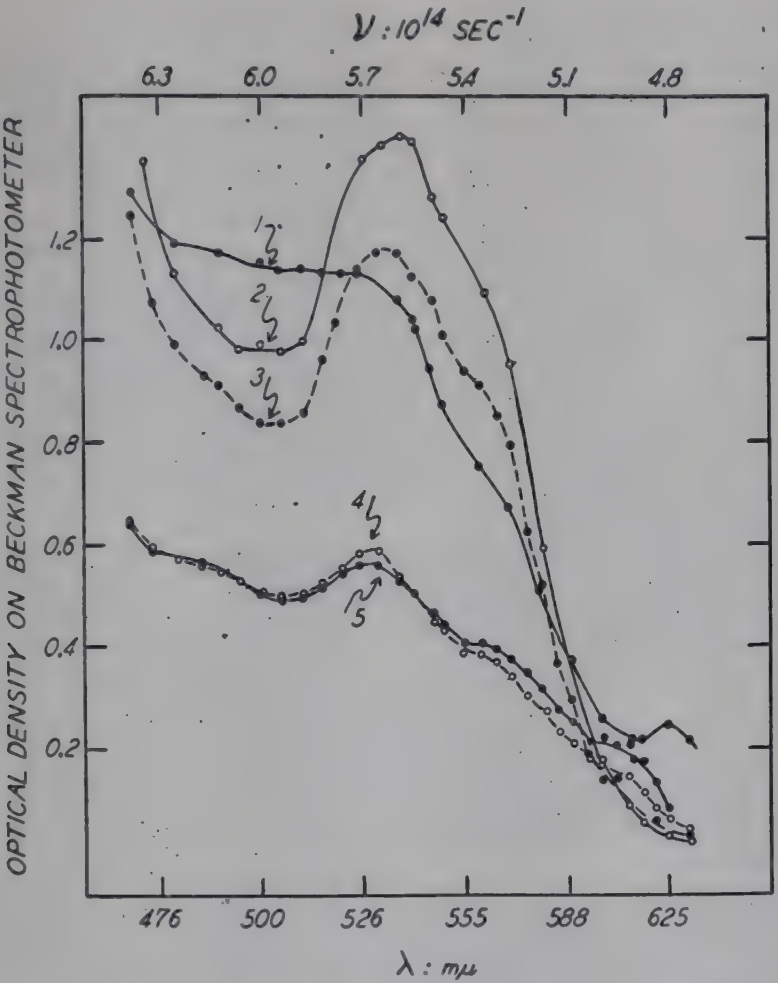

FIG. 3. Curve 1, 0.05 per cent methemoglobin in which crystalline salicylate to make a 0.5 M solution was dissolved under 680 atmospheres of pressure and the spectrogram taken at this pressure; Curve 2, solution after spectrogram of Curve 1 was taken and the pressure released; Curve 3, 0.5 M sodium salicylate solution to which methemoglobin to make 0.05 per cent was added under 680 atmospheres of pressure and the spectrogram taken at this pressure; Curve 4, 4.5 M urethane solution to which methemoglobin to make 0.05 per cent was added under 680 atmospheres of pressure; Curve 5, solution after spectrogram of Curve 4 was taken and the pressure released.

TABLE I

*Temperature for Turbidity in Presence of Various Urethanes**

	°C.		°C.
n-Butylcarbamate	38	*n*-Octylcarbamate	53
Isoamylcarbamate	40	*n*-Dodecylcarbamate	58
n-Amylcarbamate	42	Ethyl-N-phenylcarbamate	58 (slow)
n-Hexylcarbamate	43	Ethyl-N, N-diphenylcarbamate	
α-Aminopropylcarbamate	48		58 (")

* Some of these urethanes were kindly supplied by Dr. E. C. Kleiderer of the Lilly Research Laboratories and by Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc.

bidity readily developed are listed in Table I. Native methemoglobin shows turbidity at 60° only after a protracted period of time.

DISCUSSION

The similarity of the methemoglobin spectrograms in Fig. 1, Curves and 4 (urethane solution and salicylate solution), indicates that the sam molecular configuration exists in both cases. Since Anson and Mirsk have established in various ways that the protein in 0.5 M salicylate is i a proper sense denatured methemoglobin, it seems clear that urethan also denatures this protein. This result is in agreement with the observe effect of urethane in kinetic studies of luminescence, tobacco mosaic viru and invertase (2–7). Moreover, as in the previous studies with the oth systems mentioned above, the equilibrium between the protein and ur thane is markedly affected by temperature. This is in contrast to th reaction involving salicylate.

Precipitation of methemoglobin by urethane is dependent upon tem perature, with urethane concentrations less than 2.5 M. The red solutio at concentrations greater than 2.5 M becomes straw-colored on heatin but the straw color of such a solution, though reversible to red on coolin is actually not the straw color of native methemoglobin, as shown by th spectrograms of Fig. 2, Curves 1 and 3. Thus, heating does not revers the denaturation as it might appear, and a different equilibrium is ev dently concerned.

Again, unlike the effect in salicylate solutions, reversal of denaturatio does not result from dilution of a 4.5 M urethane solution of methemo globin. Rather, the molecules of the red solution react in some manne to form a protein precipitate which cannot be redissolved in saturated ur thane. The supernatant of such a precipitate has a spectrogram with contour like that of the red solution, which indicates that denaturation still complete. The reactions of methemoglobin with urethane are ob viously more complex than with salicylate.

Since pressure had no effect on the absorption of energy from the 54 mμ line by methemoglobin solutions in any concentration of sodium sal cylate, nor in 4.5 M urethane, the denaturation to this form evidently pr ceeds with no *net* volume change. If denaturation could be prevented b pressure, it would not have been complete when methemoglobin was adde under 680 atmospheres to sodium salicylate solution, as it is shown to b by the spectrogram of Fig. 3, Curve 3. What appears to be preventio of denaturation by pressure when crystalline salicylate is dissolved i methemoglobin solution under 680 atmospheres of pressure (Curve 1, Fi 3) must be the result of a complex form of salicylate in solution und pressure which is not as effective a denaturant as the salicylate form i solutions at pressures of a few atmospheres or less.

The temperatures at which the different urethanes produced turbidit as compared to the temperature at which a solution of native methem globin becomes turbid indicate that all these urethanes promote denaturation. The order of temperatures at which precipitation occurs is the order of increasing lyophobia resulting from the groups which replace the ethyl group of urethane.

SUMMARY

1. Urethane in a concentration of 4.5 M denatures methemoglobin to the same product as that caused by salicylate, but the innatured methemoglobin behaves differently with respect to temperature and to dilution of the urethane than it does with salicylate.

2. There is no net volume change in the denaturation of methemoglobin by salicylate nor by urethane of 4.5 M.

3. Each of a series of urethanes appears to be an effective denaturant, and the temperature at which denaturation is observed decreases with increasing lyophobia of the urethane derivatives, according to the substituents in place of the ethyl group of urethane.

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A STUDY OF THE METABOLISM OF DIETARY HYPOXANTHINE AND XANTHINE IN THE RAT*

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It was previously reported (1) that dietary adenine is utilized by the rat as a precursor of both the adenine and the guanine of nucleic acids. Although several compounds containing 1 to 3 carbons have been shown to be incorporated into uric acid (2, 3) or guanine (4), none of the more complex precursors of the purine ring has been identified.

The postulation that the immediate metabolic precursor of adenine is the corresponding oxygen analogue, hypoxanthine, is supported by several lines of evidence. The demonstration by Krebs *et al.* (5, 6) that pigeon liver slices synthesize hypoxanthine led him to suggest that it is an intermediate in the formation of uric acid in the pigeon. Sonne, Buchanan, and Delluva (2) have pointed out that their results are in accord with Krebs' hypothesis. Greenberg (7) has demonstrated that formic acid, which is a precursor of uric acid in the intact animal (2), is also incorporated into the hypoxanthine produced in pigeon liver homogenates. In the case of *Neurospora crassa*, forty-five adenine-requiring mutants have been characterized, and of these forty-three utilize hypoxanthine in lieu of adenine for their purine requirements (8). The interconvertibility of the 5′-nucleotides of hypoxanthine and adenine, muscle inosinic and muscle adenylic acids, has been demonstrated through the rapid uptake of isotopic NH_3 into the 6-amino group of this adenylic acid (9).

In addition, folic acid-deficient *Escherichia coli* produces (10, 11) 5(4)-amino-4(5)-imidazolecarboxamide and it has been shown (12) that glycine (or threonine) in the medium stimulates the production of this imidazole. This, in conjunction with the data showing that glycine is a specific precursor of uric acid, supports the suggestion of the latter authors that this imidazole derivative plus formic acid is the immediate precursor of hypoxanthine and thence of other purines.

If these arguments are valid and if hypoxanthine is converted to ade-

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nine by replacement of the 6-hydroxyl by an amino group, a similar
lationship might result in the amination of the 2 position of xanthi
to yield guanine.

To test these hypotheses we have prepared hypoxanthine and xa
thine, each labeled with isotopic nitrogen in the 1 and 3 positio
Hypoxanthine was first prepared by the deamination of isotopic adenin
however, the product so prepared is difficult to free from the last trac
of adenine and it was preferable to synthesize hypoxanthine indepen
ently. The sample of xanthine was prepared by deamination of isoto
guanine.

TABLE I

Feeding of Hypoxanthine and Xanthine

	Hypoxanthine, 26 mg. per kilo per day		Hypoxanthine, 200 mg. per kilo per day		Xanthine, 105 mg. per kilo per day	
	Atom per cent excess N^{15}*	Calculated on basis of 100% in compound fed	Atom per cent excess N^{15}	Calculated on basis of 100% in compound fed	Atom per cent excess N^{15}	Calcula on bas of 100% compou fed
Dietary purine	4.34	100	7.67	100	5.37	100
Nucleic acids	0.006	0.14	0.011	0.14	0.006†	0.
Purines from nucleic acids	0.002‡	0.05	0.014§	0.18	0.003§	0.
Copper purines from muscle	0.004	0.09	0.003	0.04		
Muscle protein			0.000	0.00		
Allantoin	0.333	7.7	2.95	38.5	1.32	24.
Urea	0.009	0.21	0.017	0.22	0.015	0.
Ammonia	0.011	0.25			0.029	0.
Total urinary N	0.024		0.256			

* Consolidated-Nier ratio mass spectrometer, average error ± 0.002.

† Sodium nucleates.

‡ Purine hydrochlorides.

§ Copper purines.

Hypoxanthine was fed to rats in two experiments, at levels of 26
200 mg. per kilo of body weight per day. These levels correspond o
molar basis to the two levels at which adenine was administered to
(1). The results (Table I) show that dietary hypoxanthine is not
lized as a precursor of nucleic acid purines nor of the extractable puri
of muscle. Conversion to urinary allantoin was extensive; 7.7 and 3
per cent of the urinary allantoin were derived from the labeled diet
hypoxanthine. These values are somewhat higher than those obtai
from the corresponding levels of adenine, which were 5.5 and 27.0.

The possibility remains that some derivative of hypoxanthine e involved in the elaboration of nucleic acid purines but, in the rat, he mediation of the free dietary hypoxanthine is excluded.

The labeled xanthine was fed at a level of 105 mg. per kilo of rat per day, which is 3.5 times the lower level at which adenine and hypoxanthine were administered. There was no incorporation of its isotopic nitrogen into the nucleic acids, but it also was extensively converted to allantoin.

In the case of both hypoxanthine and xanthine, little isotopic nitrogen was found in the ammonia or in the urea. The values obtained represent a slightly more significant degradation to ammonia than resulted when uric acid was fed (13), when the actual atom per cent excess N^{15} of the allantoin and the urea was 5.37 and 0.006 respectively. The fact that the ring nitrogens of these oxypurines lead to no more than traces of ammonia and urea substantiates the earlier supposition that the appreciable amount of N^{15} found in the urea after the feeding of guanine (1) was indeed derived from the labeled 2-amino group of the guanine, and not from its ring nitrogens.

EXPERIMENTAL

Hypoxanthine, from Adenine—The deamination (14) of 850 mg. of adenine labeled in the 1 and 3 positions (1) (6.4 atom per cent excess N^{15}) yielded 271 mg. of hypoxanthine. Counter-current distribution analysis (15) in *n*-butanol-1 M phosphate buffer, pH 6.5, showed that this specimen of hypoxanthine ($K = 0.55$) contained nearly 20 per cent of adenine ($K = 2.2$). These were separated by a twenty-one plate preparative distribution in *n*-butanol-water, in which the distribution constants are nearly the same as in the buffer, but from which the recovery could be accomplished more easily because of the absence of salts. 500 cc. separatory funnels were used with 200 cc. portions of each solvent in each. The contents of Funnels 14 through 19 were combined and concentrated *in vacuo* (in contrast to the situation in the "distribution machine" the lower phase was transferred from funnel to funnel, so that the more water-soluble hypoxanthine migrated more rapidly down the series of funnels). The purified hypoxanthine, 64 mg., was recovered by crystallization from water and an analytical counter-current distribution showed it to contain not more than a fraction of a per cent of adenine. 52 mg. of this product were mixed with 39 mg. of normal hypoxanthine and this sample, containing 4.34 atom per cent excess N^{15}, was fed in the first experiment.

Analysis—$C_5H_4ON_4$. Calculated,[1] N 41.3; found, N 39.7

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[1] Corrected for content of isotopic nitrogen.

Hypoxanthine, Synthesis—Thiourea, containing 16 per cent excess N was prepared (16) in 91 per cent yield from ammonium nitrate.

4,5-Diamino-6-hydroxypyrimidine hydrochloride was prepared a cording to the procedures described (17), except that, in the dethiolati 4 gm. of Raney's nickel, 1.0 gm. of anhydrous potassium carbonate, a 10 cc. of water were used for 1.85 gm. of the 4,5-diamino-6-hydroxy-thiopyrimidine. After the solution had been refluxed, Darco was add and the solution was warmed and filtered. The filtrate was cooled a saturated with dry HCl, again cooled, and the product collected. 1. gm. of product containing potassium chloride were obtained. The spe trum of the crude product agreed with that of 4,5-diamino-6-hydrox pyrimidine (18) and it was estimated that 640 mg. of the pyrimidi hydrochloride were present. A second crop was precipitated by t addition of alcohol and it contained about 630 mg. of the pyrimidine.

The closure of the imidazole ring to form hypoxanthine was accor plished by the method developed for isoguanine and 2,6-diaminopuri (19). The 1.43 gm. of crude product and 11 cc. of 1 N formic acid anhydrous formamide were heated in a sealed tube at 160° for 2 hou The tube was opened and the contents were evaporated to dryness on steam bath under a current of air. The residue was recrystallized fro 60 cc. of water, after treatment with Darco, and 310 mg., 71 per ce based upon the estimated quantity of the pyrimidine used, were o tained. The hypoxanthine contained 7.67 atom per cent excess N^{15}.

Analysis—$C_5H_4ON_4$. Calculated,[1] N 41.4; found, 41.3

Counter-current distribution analysis in *n*-butanol-1 M phosphate, p 6.6, showed a $K = 0.50$ and a homogeneity of 98 ± 2 per cent.

Xanthine—Xanthine containing isotopic nitrogen in the 1 and 3 po tions was prepared by the deamination (20) of guanine, which contain an excess of isotopic nitrogen in the 2-amino group and in the 1 and nitrogens of the ring (1). 815 mg. of xanthine containing 5.37 ato per cent excess N^{15} were obtained from 1 gm. of guanine.

Analysis—$C_5H_4O_2N_4$. Calculated,[1] N 36.8; found, N 36.1

Counter-current distribution analysis showed it to be 98 ± 2 per ce homogeneous.

Feeding Experiments—The animals used were adult male Sherm strain rats. For a few days prior to the experiment, they were fed diet consisting of 60 gm. (dry weight) per kilo of rat per day of grou pellets of Purina chow mixed with water. The urine was collected und toluene. The animals were fed the labeled compound admixed w he moistened food for 3 or 4 days. On the next day they were given ellets and were sacrificed by urethane anesthesia at the end of that day.

Hypoxanthine Feeding; Experiment I—Four rats with a combined weight of 1162 gm. were fed 91 mg. of hypoxanthine, prepared from adenine, over a 3 day period.

Experiment II—Two rats weighing a total of 430 gm. were fed 258 mg. of synthetic hypoxanthine over a period of 3 days.

Xanthine Feeding—Four rats weighing a total of 1356 gm. were fed 571 mg. of xanthine over a period of 4 days and they were sacrificed on the 5th day.

Isolations—From the urine ammonia was isolated by adsorption on permutit, urea as the dixanthydrol derivative, and allantoin via the mercury salt (1). Sodium nucleates, nucleic acids, and purine hydrochlorides (1) or copper purines (21) were isolated from the combined viscera.

In the two hypoxanthine experiments, copper purines were also isolated from the frozen muscle. The muscle was homogenized in a Waring blendor with 10 per cent trichloroacetic acid and the mixture was heated at 90° for 20 minutes. It was then strained through cheese-cloth and the residue was again extracted with hot 5 per cent trichloroacetic acid. The combined extracts were filtered through Celite by suction and the copper purines were isolated (21).

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SUMMARY

Isotopically labeled hypoxanthine and xanthine have been prepared by the deamination of labeled adenine and guanine. Labeled hypoxanthine has also been synthesized independently.

Dietary hypoxanthine and xanthine are ineffective as precursors of nucleic acids in the rat but are extensively converted to allantoin.

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HE p-PHENYLAZOBENZOYL ESTERS OF THE NATURAL ESTROGENS AND THEIR SEPARATION BY CHROMATOGRAPHIC ADSORPTION*

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The colored esters of p-phenylazobenzoic acid have been used for the hromatographic separation of sterols (1, 2), tocopherols (3), sugars (4, 5), and sugar derivatives (5–11). Bergel and Cohen (12) prepared the p-phenylazobenzoyl ester of estrone but were unable to effect a purification of the ester by chromatographing on alumina. Hydrolysis of the recrystallized ester, however, afforded a better means of purifying estrone than did recrystallization of the estrone directly from alcohol.

The possibility of separating the naturally occurring estrogenic hormones found in extracts of pregnancy urine by means of chromatographing their p-phenylazobenzoyl (abbreviated azoyl) esters was investigated. Since the azoyl esters are all highly colored, it was hoped that this method might be made the basis for a colorimetric chemical assay. The azoyl esters of six of the natural estrogens were prepared and were found to be strongly adsorbed on Florisil from an equal mixture of benzene and high boiling petroleum ether (Skellysolve C). After development and elution of the zones with increasing concentrations of ethanol in Skellysolve C, the esters were quantitatively separated into monoazoates and diazoates. A procedure, which thus far has been applied only to pure solutions, is described for the quantitative estimation of estrone and α-estradiol in a mixture. It entails (1) the quantitative preparation of the azoyl esters, (2) the removal of the excess azoyl chloride by chromatographic adsorption on alumina, (3) the separation of estrone azoate and α-estradiol diazoate by chromatographic adsorption on Florisil, and (4) the measurement of the color on the Beckman spectrophotometer.

EXPERIMENTAL

Preparation of Azoyl Esters of Six Natural Estrogens—Quantitative yields of estrone azoate, equilin azoate, equilenin azoate, α-estradiol diazoate, β-estradiol diazoate, and estriol triazoate were obtained in the

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* The material in this report was taken from a dissertation submitted by Ernest J. Umberger to the Graduate School of Georgetown University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1948.

following manner: From 10 to 30 mg. of the dry estrogen were weigh
into a dry glass-stoppered test-tube, and 100 mg. of azoyl chloride (1
and 2.0 ml. of anhydrous pyridine (purified by oxidation with ceric sulfa
according to the method of Wilson and Hughes (14)) were added. T
tube was stoppered, the stopper secured with a holder, and the tube heat
in the steam bath for 2 hours. Immediately upon removal from the stea
bath, the contents of the tube were diluted with 5.0 ml. of anhydro
pyridine and 10 ml. of a solution of 5 per cent pyridine in benzene. T
excess azoyl chloride was removed by passing the solution through a ch
matographic column containing 10 gm. of activated alumina wet with
ml. of 5 per cent pyridine in benzene. The ester was completely remov
from the column by washing with 30 ml. of 5 per cent pyridine in benze

TABLE I

Melting Points of p-Phenylazobenzoyl Esters of Several Natural Estrogens

Ester	M. p.
	°C.
Estrone azoate*	221.5–223.5
Equilin "	243 –248
Equilenin azoate	251 –253
α-Estradiol diazoate	186.5–187.5
" diazoate·H_2O†	152 –153
β-Estradiol diazoate	174 –175
" diazoate·H_2O†	124 –126
Estriol triazoate	138 –140

* Bergel and Cohen (12) found the melting point of estrone azoate to be 226.
227.5° (corrected) when recrystallized from ethyl acetate.

† Recrystallized from a dioxane-water mixture. All the other compounds we
recrystallized from a mixture of carbon disulfide and absolute alcohol.

The solvent was removed either by distillation or by evaporation on t
steam bath in a small beaker under a current of air. The yields we
always slightly greater than 100 per cent owing to a small amount of i
purity formed in a side reaction between the pyridine and the azoyl ch
ride. The esters were recrystallized from a mixture of carbon disulfi
and absolute ethyl alcohol and their melting points determined by use o
short stem thermometer. The results are shown in Table I. The est
diol diazoates contained 1 molecule of water of crystallization when
crystallized from a dioxane-water mixture. The composition of the est
was checked by a nitrogen determination.[1]

The esters are all orange to red-colored solids, generally soluble in b zene, pyridine, dioxane, chloroform, carbon disulfide, and acetone, but nsoluble, or very sparingly soluble, in petroleum ether, ethyl ether, nitromethane, ethanol, and methanol.

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[1] Nitrogen was determined by HI reduction followed by the Kjeldahl meth
The authors are indebted to Mrs. Dorris C. Chambers, Division of Allergen Inve
gations, United States Department of Agriculture, for these determinations.

Spectral absorption curves showed the typical absorption for the azo grouping with a maximum absorbency at 450 mμ, the intensity of the absorption being proportional to the number of azo groupings present.

Behavior of Estrogen Azoates on Chromatographic Adsorption Columns—In order to select a combination of adsorbent and solvents for the chromatographic separation of estrone azoate and α-estradiol diazoate, a large number of trials were made by dissolving a few crystals of each ester in a non-polar solvent and passing the solution through a column of adsorbent approximately 1 cm. in diameter and from 10 to 15 cm. long. When adsorption occurred, the column was washed with more of the same solvent and, in general, the zone moved down the column very slowly. To develop the chromatogram and to hasten elution of one of the fractions, a more polar solvent was added to the original solvent. A partial list of the combinations tried is shown in Table II. From the general appearance of the zones on the column, it seemed as though the separation of estrone azoate and α-estradiol diazoate might be accomplished by adsorption on alumina with benzene and ethanol as the solvents, on Florisil with Skellysolve C and ethanol or methanol, on a mixture of silicic acid and Filter-Cel with carbon disulfide and nitromethane, or on a mixture of Silene EF and Filter-Cel with Skellysolve C and ethanol. Adsorption of the esters on alumina from a benzene solution and elution with a mixture of benzene and ethanol failed to give a quantitative separation, since both zones appeared to be eluted as mixtures. A quantitative separation has been achieved by adsorption on Florisil from an equal mixture of benzene and Skellysolve C and elution with Skellysolve C and ethanol.

The anomalous effect described by Schroeder (15) as "double zoning" has been observed also with the estrogen azoates. For example, when α-estradiol diazoate, which appeared from other criteria to be pure, was dissolved in a mixture of equal volumes of benzene and Skellysolve C and passed over Florisil, all of the ester was adsorbed in a narrow zone at the top of the column. On development with 0.25 per cent ethanol in Skellysolve C, two well separated zones were formed. When these zones were eluted and rechromatographed, both again produced two zones on the column. Materials isolated from both zones appeared to be identical. This phenomenon was found to occur in every case in which a separation of estrone azoate and α-estradiol diazoate could be made. In spite of the appearance of two zones on the column, on continued elution all of the material was eluted as a single zone with very long tailing, as demonstrated by an elution curve (Fig. 1).

No conditions have been found for the separation of the diazoates of

Table II

Survey of Adsorbents and Solvents for Chromatographic Separation of Estrone Azoa and α-Estradiol Diazoate

Adsorbents*	Solvents		Observations
	Non-polar	Polar	
Alumina	Benzene	Ethanol	Adsorbed; separation indicated
	“	Methanol	“ “ “
	Ethylene dichloride		Not adsorbed
Florisil	Benzene	Ethanol	Adsorbed; separation indicated
	“	Methanol	“ “ “
	Skellysolve C	Ethanol	“ “ “
	“ “	Methanol	“ “ “
	“ “	Ether	“ “ “
	“ “	Nitroethane	“ “ “
	Ethylene dichloride		“ “ “
	Carbon disulfide		“ “ “
Silicic acid + Celite	Skellysolve C	Ethanol	“ no separation
	Ethylene dichloride		“ separation indicated
	Carbon disulfide	Ethanol	“ no separation
	“ “	Methanol	“ “ “
	“ “	Acetone	“ “ “
	“ “	Nitromethane	“ separation indicated
	“ “	Nitroethane	“ “ “
	Chloroform	Methanol	“ “ “
	“	Nitromethane	“ no separation
Magnesium oxide + Celite	Skellysolve C	Ethanol	“ “ “
	Ethylene dichloride		Not adsorbed
Calcium oxide	Carbon disulfide		“ “
Calcium carbonate	Ethylene dichloride		“ “
Silene EF + Celite	Skellysolve C	Ethanol	Adsorbed; separation indicated

* The adsorbents were obtained from the following sources: alumina, activate alumina, Alorco F-20 (80 to 100 mesh), Aluminum Ore Company, East St. Loui Illinois; Florisil, 100 to 200 mesh, Floridin Company, Inc., Warren, Pennsylvani silicic acid, Mallinckrodt, precipitated; Celite, No. 535, Johns-Manville; ma nesium oxide, Mallinckrodt, analytical reagent; calcium oxide, analytical reagen ignited, General Chemical Company, New York; calcium carbonate, analytic reagent, General Chemical Company, New York; Silene EF, courtesy of Pittsburg Plate Glass Company, Columbia Chemical Division, Barberton, Ohio.

-estradiol and β-estradiol, nor have the azoates of estrone, equilin, and quilenin been separated. It appears that only a separation of the mono-zoates from the diazoates can be accomplished. The diazoates form the ower zone on the column and are eluted first. A few trials with estriol triazoate indicate that its adsorption properties are similar to those of the monoazoates. α-Estradiol diazoate can be separated from estriol triazoate on the Florisil or silicic acid columns, but estrone azoate and estriol triazoate form continuous chromatograms.

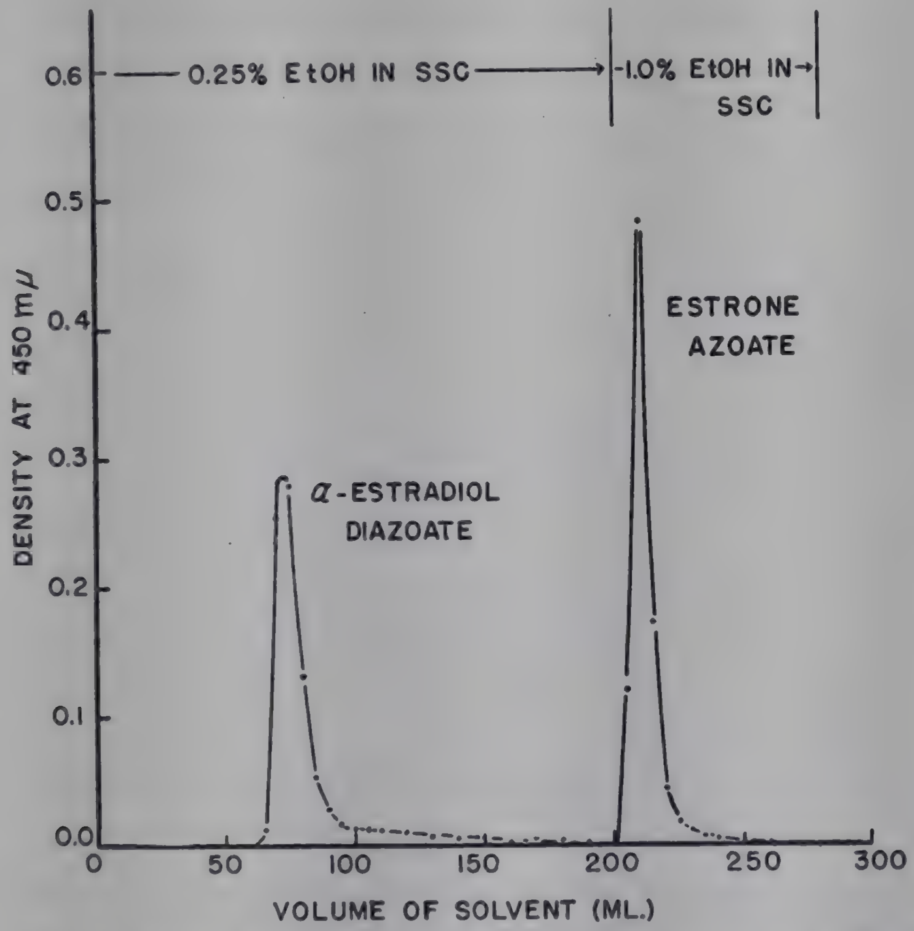

Fig. 1. Elution curve for a mixture of α-estradiol diazoate and estrone azoate adsorbed on Florisil and eluted with Skellysolve C and ethanol. Amount of Florisil, 5.0 gm. The eluate was collected in 5 and 10 ml. lots, and the optical density of each was measured on the Beckman spectrophotometer. The α-estradiol diazoate was eluted with 200 ml. of 0.25 per cent ethanol in Skellysolve C and the estrone azoate with 80 ml. of 1.0 per cent ethanol in Skellysolve C.

Quantitative Separation of Azoyl Esters of Estrogens into Monoazoates and Diazoates—The separation of estrone azoate and α-estradiol diazoate may be used as an example. The column was prepared by sealing a glass tube about 20 cm. long and of 10 mm. outside diameter to the outer part of a standard taper 24/40 joint. The lower end was drawn down to about

3 mm. inside diameter and a wad of cotton used to hold the adsorbe The standard taper joint provided a chamber for holding about 25 ml. solvent and could be connected to an outlet for applying pressure. Abo 5.5 gm. of Florisil (100 to 200 mesh) was slurried into the column with ml. of 5 per cent ethanol in Skellysolve C. The use of ethanol at th stage prevented the adsorbent from sticking to the sides in the upper pa of the column. Air pressure of about 500 mm. of mercury was appli and the column gently tapped with a wooden stick while the adsorbe settled. The column was then washed with 25 ml. of Skellysolve C remove the alcohol. A solution of 5.7 mg. of α-estradiol diazoate and 2 mg. of estrone azoate, dissolved in 10 ml. of an equal mixture of benze and Skellysolve C, was passed through the column without pressure. Th esters were washed in with 10 ml. of Skellysolve C. All of the color wa adsorbed in a zone about 1.8 cm. wide at the top of the adsorbent. Development with 0.5 per cent ethanol in Skellysolve C caused separatio into two zones and the elution of the lower zone. The first 40 ml. of fi trate were discarded and the second 40 ml. containing the lower zone wer collected as Fraction 1. Elution of the second zone was accomplishe with 40 ml. of 2.0 per cent ethanol in Skellysolve C and was collected a Fraction 2. After evaporation of the solvent, the residue from Fraction weighed 5.7 mg. and melted without recrystallization at 186–188°. Th residue from Fraction 2 weighed 2.5 mg. and melted without recrystallization at 212–214°. Quantitative recovery of the esters approximatin chemical purity was, therefore, obtained.

Determination of Estrone and α-Estradiol in Mixtures in Pure Solutio by Means of Their Colored Azoyl Esters—Based on the foregoing, an experiment was carried out in which the components of various mixture of estrone and α-estradiol were determined. Separate solutions of estron and α-estradiol were prepared in absolute alcohol containing 1.0 mg. the estrogen per ml. In six reaction tubes were added the appropriat amounts of the alcohol solutions to give the amounts of estrogens listed i Table III. The alcohol solvent was evaporated to dryness on the stea bath under a current of air. To each tube was added 1.0 ml. of purifie pyridine and the solution again evaporated to dryness. The tubes an stoppers were then dried over phosphorus pentoxide under a vacuum fo 1 hour. To each tube were added 100 mg. of azoyl chloride and 2.0 m of anhydrous purified pyridine, the stoppers secured in place, and th tubes heated in the steam bath 2 hours. Immediately upon remova from the steam bath, the contents of each tube were diluted with 5.0 m of pyridine and 10 ml. of 5 per cent pyridine in benzene. For removal the excess azoyl chloride, a chromatographic adsorption tube 20 mm. × 200 mm. was used. 10 gm. of activated alumina were poured into th tube under suction, the suction released, and the adsorbent wet with 10 ml. of 5 per cent pyridine in benzene. The sample solution was poured through and the reaction tube washed out with 30 ml. of 5 per cent pyridine in benzene and added to the column in small portions. The ester solution was then distilled to dryness under a vacuum, air being drawn over the residue through the inlet tube at the end to remove the last traces of pyridine.

The ester residue was dissolved in 5 ml. of benzene and 5 ml. of Skellysolve C. This solution was then passed through the Florisil column described above, except that only 5.0 gm. of Florisil were used. After the solution was washed in with 10 ml. of Skellysolve C, the column was developed with 125 ml. of 0.125 per cent ethanol in Skellysolve C. This caused the elution of a reagent impurity zone and caused the estrone azoate and α-estradiol diazoate to separate into distinct zones. A small amount of pressure was applied so that the flow of solvent from the column was between 50 and 60 drops per minute. The receivers were changed and the α-estradiol diazoate fraction eluted with 0.25 per cent ethanol in Skellysolve C. The amount of this solvent was governed by the progress of the estrone azoate zone, the receivers being changed when it appeared that the front boundary of the estrone azoate zone had reached the end of the column. Between 185 and 235 ml. were required. The estrone azoate zone was then eluted with 80 ml. of 2.0 per cent ethanol in Skelly-

TABLE III

Data on Determination of Estrone and α-Estradiol in Mixture in Pure Solution by Means of Their Colored Azoyl Esters

Sample No.	α-Estradiol added	Estrone added	Optical density				Total optical density; diazoate + monoazoate fraction		
			Diazoate fraction		Monoazoate fraction				
			Found*	Expected†	Found*	Expected†	Found*	Expected†	Difference
	mg.	*mg.*							
1	0.00	0.75	0.006	0.000	0.421	0.423	0.427	0.423	+0.004
2	0.15	0.75	0.162	0.161	0.432	0.423	0.594	0.584	+0.010
3	0.30	0.50	0.322	0.322	0.279	0.282	0.601	0.604	−0.003
4	0.45	0.25	0.480	0.483	0.141	0.141	0.621	0.624	−0.003
5	0.45	0.00	0.435	0.483	0.051	0.000	0.486	0.483	+0.003

* These values are corrected for a blank in which no estrogens were added and which gave optical densities of 0.016 for the diazoate fraction and 0.011 for the monoazoate fraction.

† The optical density expected was calculated on the basis that the slope for α-estradiol is 0.161 per 0.15 mg. (or 1.073 per mg.) and the slope for estrone is 0.141 per 0.25 mg. (or 0.564 per mg.).

solve C. The fractions, which had been collected in 250 ml. round botto distilling flasks, were distilled to dryness under a vacuum and the res dues were dissolved to 5.0 ml. in benzene. The color (optical density) each fraction was measured on the Beckman spectrophotometer at 4 mμ. The results are presented in Table III. Previous experiments ha shown that the optical density to be expected for 0.15 mg. of α-estradi was 0.161 and for 0.25 mg. of estrone, 0.141. Inspection of the result in Table III shows that quantitative results were obtained with estron alone or in mixtures (Samples 1 to 4) but that the results for α-estradi alone (Sample 5) were about 10 per cent low. As shown in the last thre columns of Table III, the total color obtained was quantitative.

Hydrolysis of Estrogen Azoates and Recovery of Free Estrogens—Sinc the colors of the azoyl esters are qualitatively the same, the compositio of the various zones on the columns was determined by hydrolyzing th esters and identifying the free estrogens.

The hydrolysis procedure developed was as follows: 2 ml. of the fina benzene solution obtained above were placed in a small flask and evap orated to dryness on the steam bath under a current of air. The flas was dried over phosphorus pentoxide for 1 hour. 2 ml. of a solution c 2 per cent NaOH in absolute methanol were added, the flask attached t a reflux condenser fitted with a drying tube, and the mixture refluxe ½ hour. The cooled solution was transferred to a separatory funnel wit 25 ml. of water-washed ether and 10 ml. of 5 per cent aqueous NaHCO After shaking and allowing the solution to stand, the aqueous layer wa transferred to a second funnel containing 25 ml. of water-washed ethe shaken, and discarded. The ether solutions were then washed consecu tively with 5 ml. of 5 per cent aqueous $NaHCO_3$, and 10, 5, and 5 m portions of distilled water. The ether solutions were then distilled t dryness, the ether in the second funnel being used to wash out the fir funnel. The colorless residue was dissolved to 5 ml. in absolute alcoho An aliquot of this solution was used for quantitative determination an identification of the free estrogen by means of the sulfuric acid reactio The details of this method are described in the following paper. Quant tative recoveries of the free estrogens were obtained by the hydrolysis pr cedure described.

DISCUSSION

Conditions have been found only for the separation of the azoyl este of the natural estrogens into monoazoates and diazoates. The separ tion is similar, therefore, to the separation of the estrogens into keton and non-ketonic fractions by the well known Girard's Reagent T. Th chromatographic adsorption technique, however, may result in a mo rified product which will give less interference in the application of color actions, such as the sulfuric acid reaction or the Kober test. It has been und, for example, that cholesterol azoate is only weakly adsorbed on lorisil from a mixture of benzene and Skellysolve C and is completely moved from the column by continued washing with the same solvent, hereas the estrogen azoates are strongly retained.

In the quantitative separation of the monoazoates and the diazoates by he chromatographic adsorption method, it is important that the elution f the diazoates he continued until it appears that the monoazoate fraction is ready to begin elution, because of the very long tailing exhibited y the diazoate fraction. The appearance of two zones on the column loes not necessarily indicate that both diazoates and monoazoates are resent. In the absence of the monoazoates, however, the upper zone gradually disappears. This anomalous effect, in which pure compounds appear to give a heterogeneous chromatogram, has been termed "double zoning" by Schroeder (15).

In the development of the esterification procedure, several factors had to be taken into consideration. A procedure was desired which would allow the quantitative recovery of the esters yielded by 1 mg. or less of free estrogens. Since it was planned to measure the amount of ester colorimetrically, the blank color should be as small as possible. It was found that ordinary anhydrous pyridine contained substances which reacted with azoyl chloride to give considerable color. These interfering substances are practically eliminated by treating the pyridine with ceric sulfate. The remaining color due to these interferers appears on the Florisil chromatographic column as a reagent impurity zone preceding the diazoate zone, as blanks in the diazoate and monoazoate fractions (Table III), and as a zone retained at the top of the column after elution of the esters.

When alcoholic solutions of the estrogens were used, the alcohol was evaporated on the steam bath under a current of air. To remove the alcohol from the residue completely, pyridine was added and the pyridine evaporated.

In order to prevent the excess azoyl chloride from precipitating on removal of the reaction flask from the steam bath, 5.0 ml. of anhydrous pyridine were added to the reaction mixture. After further dilution with 5 per cent pyridine in benzene, the excess azoyl chloride was removed quantitatively from the ester solution by passing the solution through a column of alumina.

SUMMARY

A method is described for the quantitative esterification of the natural estrogens with *p*-phenylazobenzoyl chloride in small amounts. The estro-

gens and *p*-phenylazobenzoyl chloride are dissolved in a small amount anhydrous pyridine purified by oxidation with ceric sulfate, and heate on the steam bath for 2 hours. The excess azoyl chloride is remove by chromatographing on activated alumina.

The *p*-phenylazobenzoyl esters of estrone, equilin, equilenin, α-estradio β-estradiol, and estriol have been prepared and characterized by thei melting points.

The estrogen azoates may be separated quantitatively into monoazoate and diazoates by chromatographing on Florisil from an equal mixture o benzene and Skellysolve C. They are developed and eluted with smal amounts of ethanol dissolved in Skellysolve C.

The method of esterification and chromatographic separation has bee used for the quantitative determination of estrone and α-estradiol in mixtures in pure solution in less than mg. amounts.

A method for the hydrolysis of the estrogen azoates and quantitativ recovery of the free estrogens has been described.

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THE USE OF THE SULFURIC ACID REACTION FOR THE ESTIMATION OF α- AND β-ESTRADIOLS AND OF ESTRONE AND EQUILIN IN BINARY MIXTURES IN PURE SOLUTIONS*

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The problem of estimating quantitatively the components of a mixture of naturally occurring estrogens has been attacked through the preparation of their *p*-phenylazobenzoyl (azoyl) esters and separating these esters by the chromatographic adsorption technique (1). By this method, the monoazoates were quantitatively separated from the diazoates. Hydrolysis of these esters and suitable extraction procedures resulted in the quantitative recovery of the free estrogens. The present report describes a study of a single phase sulfuric acid reaction for the further analysis of the mixtures of free estrogens so obtained and for the positive identification of a single free estrogen.

It was early observed that, when the natural estrogens are treated with sulfuric acid, orange-colored solutions possessing a green fluorescence are obtained (2–4). Kober (4) showed that the initial orange color obtained with estrone was changed to a clear green-fluorescing red on warming with water, and, in addition, that the intensity of the red color could be enhanced and the intensity of the yellow color and the fluorescence diminished by using a mixture of phenol and sulfuric acid in the initial stage of the reaction instead of sulfuric acid alone. Since the orange or yellow solutions resulting from the action of sulfuric acid on cholesterol, bile acids, pregnanediol, and many other steroids are decolorized by dilution with water, the test was claimed to be specific for the natural estrogens. Cohen and Marrian (5), however, found that the presence of either cholesterol or pregnanediol considerably decreased the intensity of the red color yielded by given quantities of estrone or estriol in the Kober reaction. They resorted, therefore, to an alkali extraction procedure which separated the phenolic estrogens from these interferers, and which, in effect, supplied th specificity claimed by the Kober reaction.

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* Some of the material in this report was presented at the Thirty-second annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1948. An abstract has been published (*Federation Proc.*, 7, 261 (1948)).

This report is from a dissertation submitted by Ernest J. Umberger to the Graduate School of Georgetown University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1948.

Various modifications of the Kober reaction designed to eliminate th yellow color and the fluorescence and to increase the specificity and sensitivity of the reaction have been published (5–11). Carol and Molitor (12 have described a modification of the Kober reaction which permits the determination of the components of a binary mixture of α- and β-estradiols

Mather (13) pointed out that the spectral absorption curves of the color formed with the estrogens by phenol reagents are basically the same a those with sulfuric acid alone, and Cohen and Bates (14) have publishe the details of a method utilizing the two-stage Kober test without the us of phenol. Bates and Cohen (15) and Jailer (16) have described method utilizing the fluorescence that develops with sulfuric acid for the quantitative determination of the estrogens, and Dr. Willard M. Allen, in unpublished work made known to the authors, has made use of the yellow component of the color produced by sulfuric acid and alcohol for the quantitative determination of the estrogens in human pregnancy urine.

EXPERIMENTAL

Behavior of Natural Estrogens with Various Concentrations of Sulfuric Acid

Solutions containing 30, 45, 60, 75, 90, and 100 per cent concentrated sulfuric acid were prepared by diluting a measured amount of concentrated sulfuric acid (Baker's Analyzed, c.p., assay 95.5 to 97.5 per cent) to volume in a volumetric flask with small amounts of distilled water, and cooling after each addition. For example, 90 per cent sulfuric acid was prepared by placing 90 ml. of concentrated sulfuric acid in a 100 ml. volumetric flask and diluting to the mark with distilled water. The reaction tubes were prepared by sealing off the end of the outer part of a 24/40 standard taper joint and closing with a 24/40 standard taper penny head stopper. To the tube was added an alcoholic solution of 25 γ of the estrogen and the alcohol was evaporated on the steam bath under a stream of air. The tube and stopper were dried over phosphorus pentoxide under a vacuum for 1 hour. The tube was then placed in an ice bath, 5.0 ml. of the diluted sulfuric acid were added, and the tube was stoppered, allowed to remain in the ice bath 5 minutes, and heated in a vigorously boiling water bath. During the heating period, the tube, without removal from the bath, was agitated at 15 second intervals during the 1st minute and at 1 minute intervals thereafter. On removal from the boiling water bath the tube was again placed in the ice bath for 1 minute. The absorption spectrum of the color produced was measured in the range from 400 to 560 mμ on the Beckman spectrophotometer.

Absorption curves were determined for α-estradiol (Fig. 1), β-estradiol Fig. 2), estrone (Fig. 3), and equilin (Fig. 4), after a 12 minute heating period. This heating period was chosen since α-estradiol and β-estradiol give equal intensities of absorption at 515 mμ after 12 minutes heating. It will be observed that with 60 per cent sulfuric acid the curves for all four estrogens are similar. The solutions are orange in color and fluoresce strongly. Two peaks of maximum absorption are shown, one in the neighborhood of 460 mμ and one in the neighborhood of 515 mμ. As the sulfuric acid concentration is increased, the solutions become yellow, with an increase in absorption at the lower wave-length relative to the absorption at the higher wave-length. The converse is true on decreasing the sulfuric acid concentration, and the solutions become red. Also as the concentration of sulfuric acid is increased, the absorption maxima are shifted towards the lower wave-lengths in the cases of α-estradiol, β-estradiol, and estrone. With equilin, however, these maxima become less distinct, there being general absorption throughout the region of the spec-

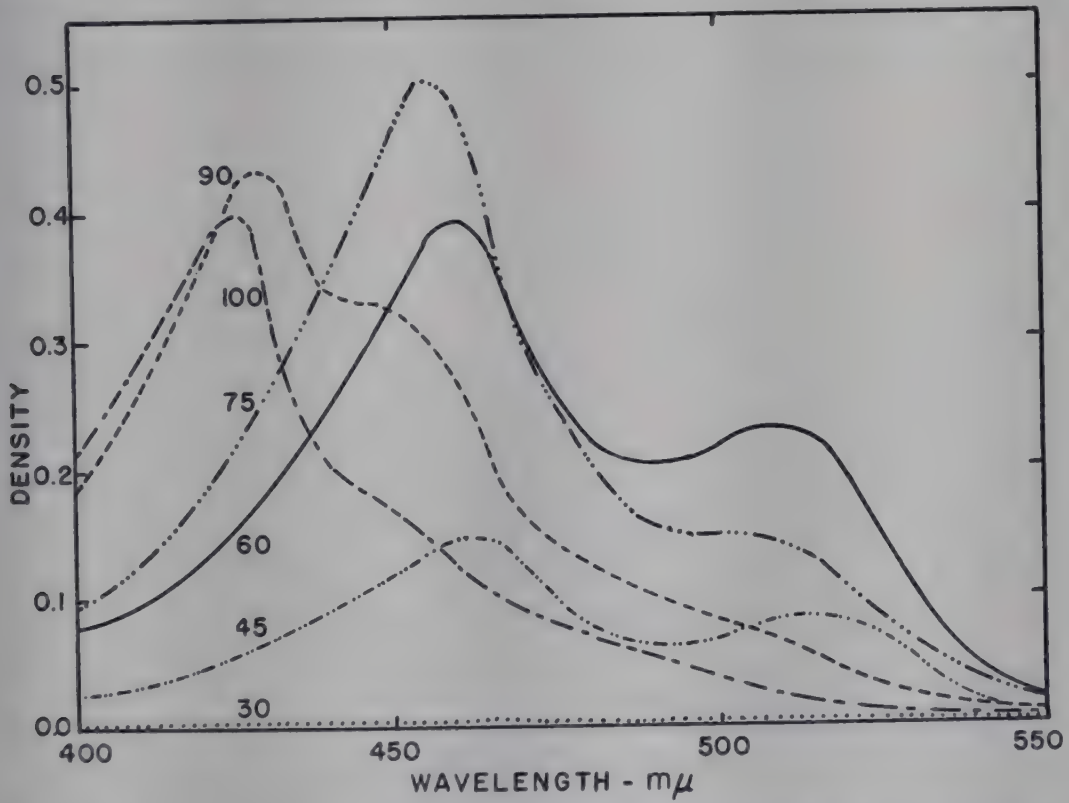

FIG. 1. Effect of concentration of sulfuric acid on spectral absorption given by 25 γ of α-estradiol when heated with 5.0 ml. of various concentrations of sulfuric acid for 12 minutes. The Beckman spectrophotometer, which was used for the determination of the spectral absorption curves, employs a 1 cm. cell containing about 4 ml. of solution. Readings may be made as per cent transmission (T) or as optical density (D). They may be readily converted by the relation, $D = 2 - \log_{10} T$.

trum observed. With very high concentrations of sulfuric acid, α-estr diol exhibits a new maximum at 425 to 430 mμ. This new maximu appears to be specific for α-estradiol, although it is faintly evident in th cases of β-estradiol and equilin with 100 per cent sulfuric acid.

The changes in absorption intensity at 460 and 515 mμ with concentra tion of sulfuric acid for the estradiols are shown in Fig. 5. There is clearl an optimal concentration of sulfuric acid for both wave-lengths which however, differs for the two isomers. β-Estradiol reacts more strongly a the lower concentrations, while α-estradiol reacts more strongly at th higher concentrations. Equal intensities of absorption were given with 60 per cent sulfuric acid at 515 mμ and with 73.5 per cent sulfuric acid at 460 mμ. β-Estradiol is the only estrogen so far tested which shows appreciable reaction with sulfuric acid concentrations of 30 per cent or lower. No conditions were found at which the α isomer reacts exclusively.

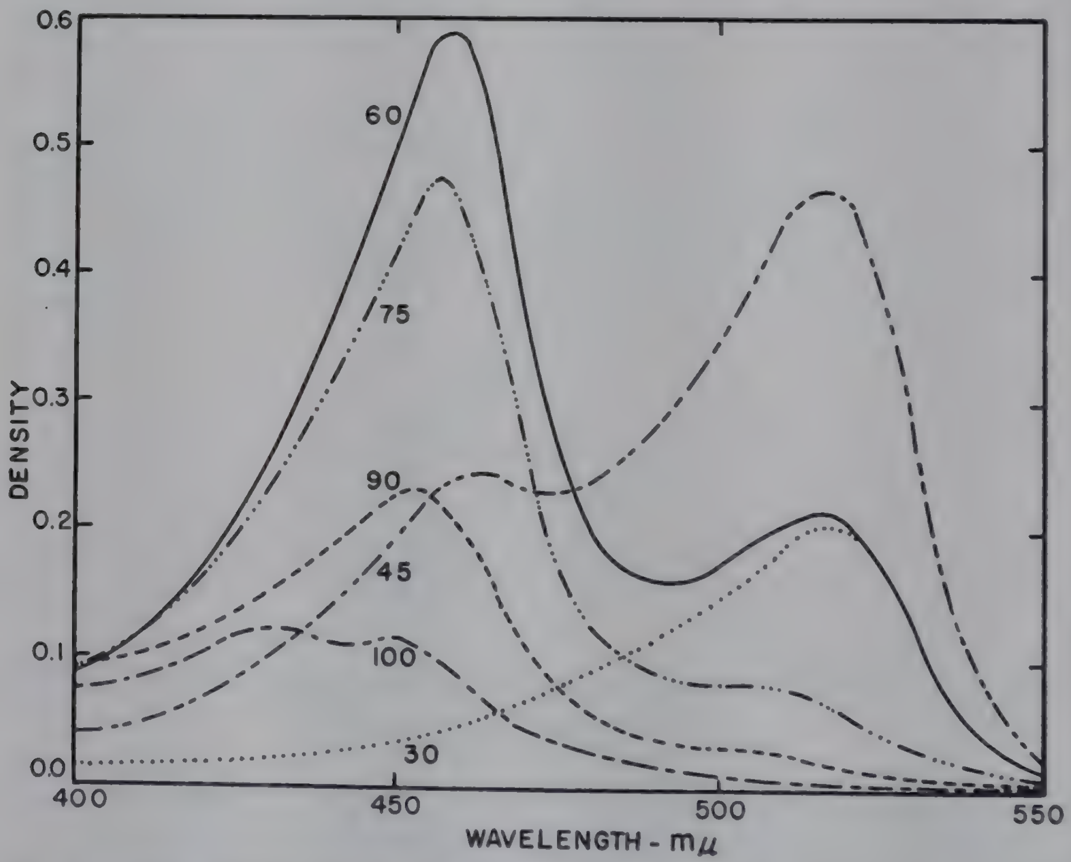

Fig. 2. Effect of concentration of sulfuric acid on spectral absorption given by 2 γ of β-estradiol when heated with 5.0 ml. of various concentrations of sulfuric acid for 12 minutes.

Based on the above observations, methods were developed for the quantitative estimation of α-estradiol and β-estradiol in a binary mixture, and of estrone and equilin in a binary mixture. Methods could probably be developed for determining any two of the estrogens in a binary mixture.

Determination of β-Estradiol

In an attempt to determine β-estradiol quantitatively in the presence f α-estradiol with 30 per cent sulfuric acid as the reagent, cloudy solutions leveloped owing to the lower solubility of the α isomer. This condition vas remedied by the addition of *n*-butyl alcohol to 20 per cent by volume. With this 30 per cent sulfuric acid-20 per cent *n*-butyl alcohol reagent,

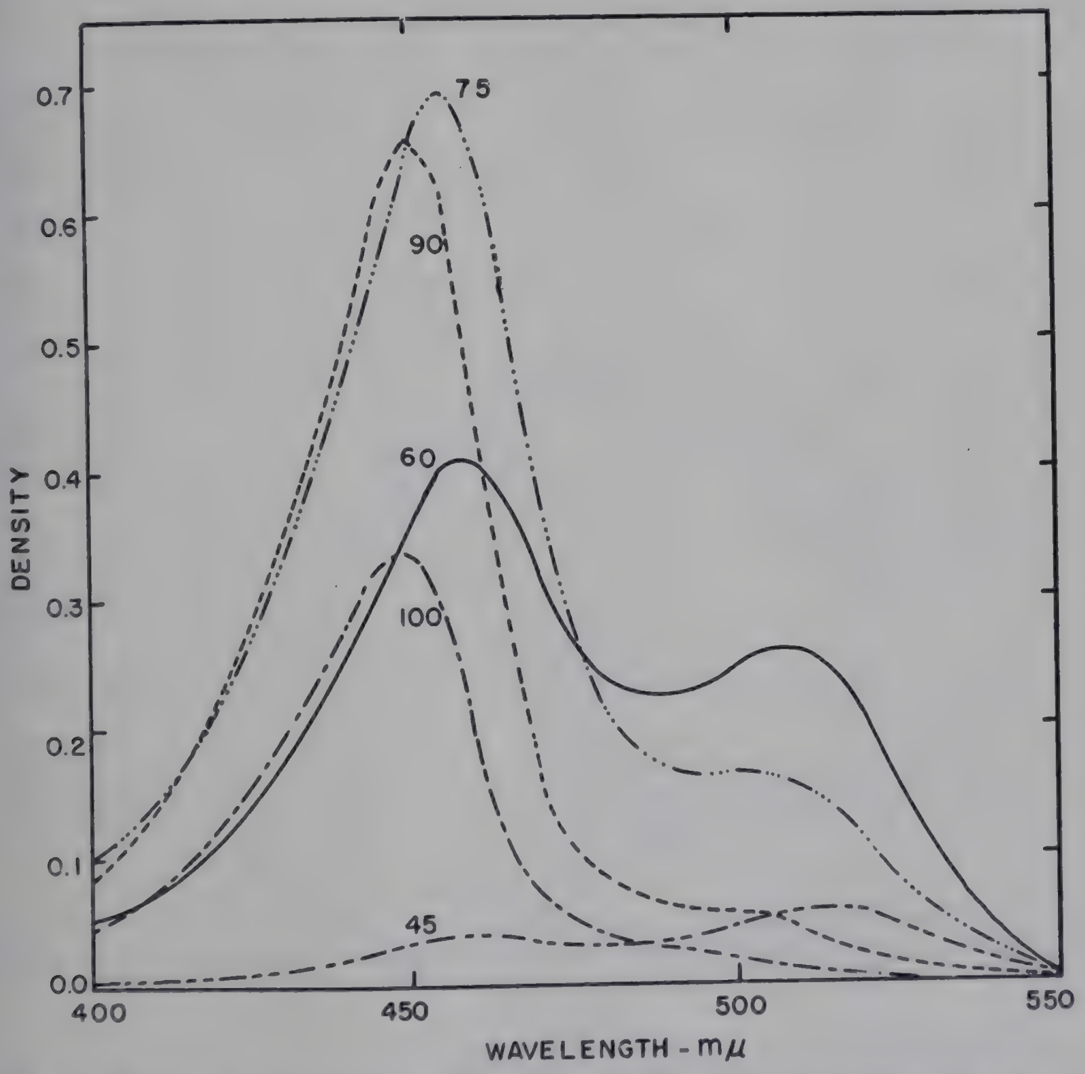

FIG. 3. Effect of concentration of sulfuric acid on spectral absorption given by 25 γ of estrone when heated with 5.0 ml. of various concentrations of sulfuric acid for 12 minutes.

optimal conditions were obtained when the time of heating was 6 minutes and the readings were made at 524 mμ. The color was stable for at least 1 hour. Fig. 6 shows the results of a series of determinations of β-estradiol under these conditions. Beer's law is followed up to 50 γ. Fig. 6 indicates that on addition of 100 γ of α-estradiol to the β-estradiol there is no interference by this compound. Estrone, equilin, α-dihydroequilin, equilenin, and estriol fail to give a color under these conditions in amounts of 50 to 100 γ. Other dihydro compounds have not yet be tested.

Determination of Total Estradiols

Since no conditions were found in which α-estradiol reacted exclusivel a method was developed for determining total estradiols. α-Estradi can then be determined indirectly as the difference between the value f total estradiols and the value for β-estradiol.

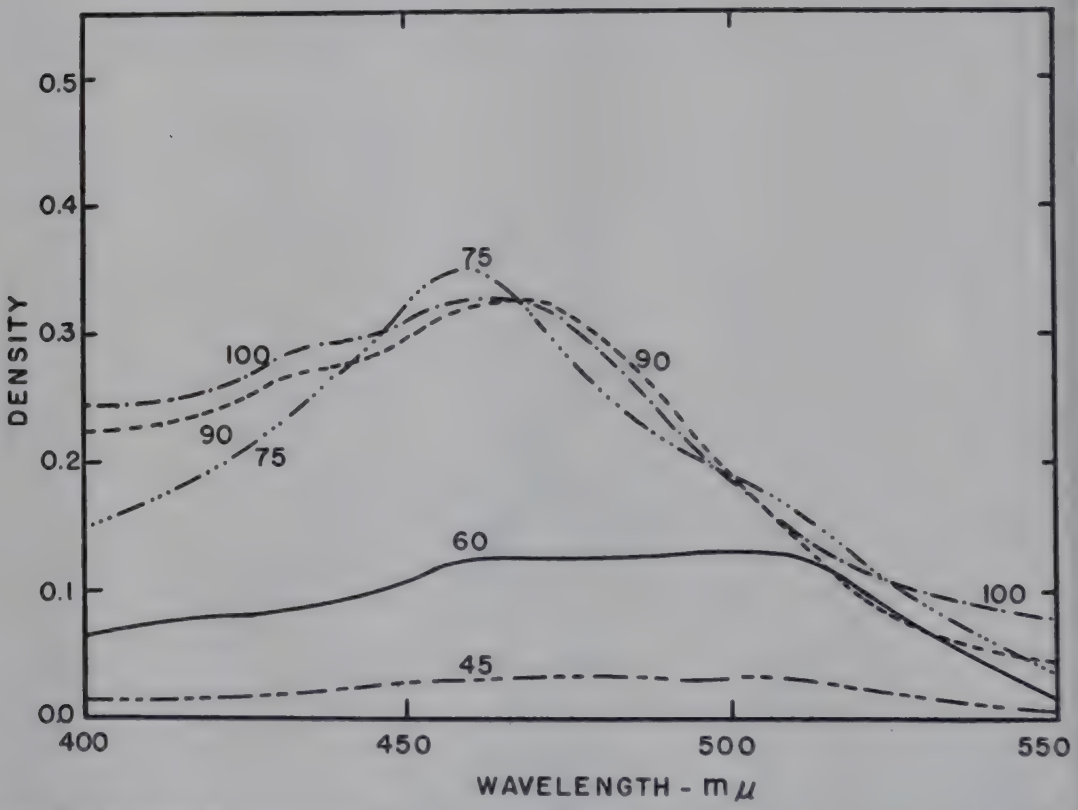

FIG. 4. Effect of concentration of sulfuric acid on spectral absorption given by γ of equilin when heated with 5.0 ml. of various concentrations of sulfuric acid for minutes.

While the α- and β-estradiols gave equal intensities of absorption 515 mμ when heated with 60 per cent sulfuric acid for 12 minutes, the was an enhancement of the absorption when the two were mixed, resulti in an overestimation of total estradiols of from 5 to 20 per cent.

On the other hand, total estradiols were satisfactorily determined heating with 5.0 ml. of 73.5 per cent sulfuric acid for 12 minutes. Und these conditions, the maximum sensitivity was obtained by reading at 4 mμ. There appeared to be no tendency towards either enhancement inhibition of absorption in mixtures. Spectral absorption curves for t two isomers were practically identical in the region from 400 to 470 r ut α-estradiol gave greater absorption in the region from 470 to 560 mμ, s would be predicted by the curves in Fig. 5.

imultaneous Determination of Estrone and Equilin in Binary Mixture

Since there was considerable difference in the spectral absorption curves iven by estrone and equilin when heated with 90 per cent sulfuric acid, hey could be determined simultaneously in mixtures by reading at two

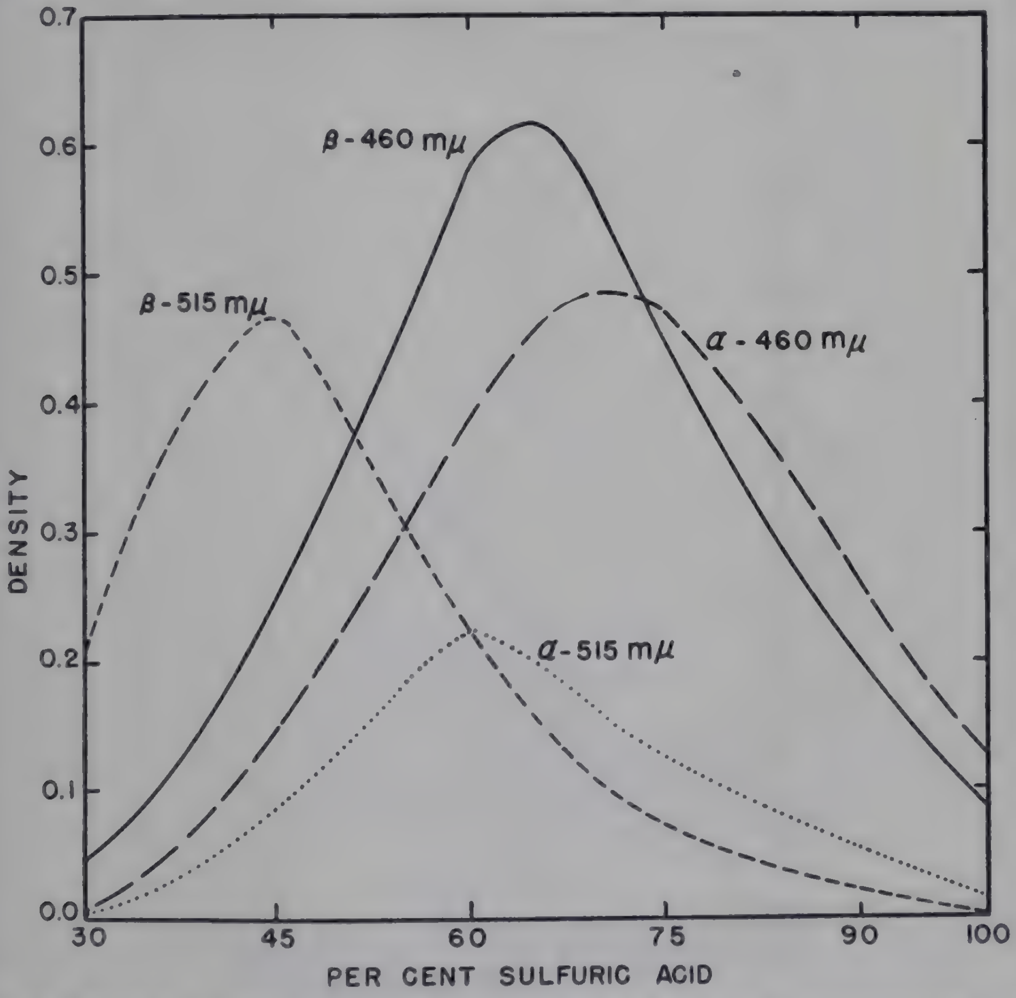

FIG. 5. Variation of the absorption at 460 and 515 mμ with concentration of sulfuric acid for α-estradiol and β-estradiol (25 γ of each heated with 5.0 ml. of diluted sulfuric acid for 12 minutes).

wave-lengths and solving simultaneous equations to obtain the concentration of each component. General formulas for such a calculation are given by Knudson, Meloche, and Juday for the simultaneous determination of iron and aluminum in water by the hematoxylin method (17). This method can be used only when there is no appreciable interference between the development of colors by the two components. To test this, estrone and equilin were heated separately and in an equal mixture with 5.0 ml. of 90 per cent sulfuric acid for 5 minutes and the spect absorption curves determined. A 5 minute heating period was us instead of a 12 minute period to reduce the amount of color due to charri which might be expected in an impure solution. The curves so obtain are shown in Fig. 7. Curve A is the absorption curve of 20 γ of equil alone and Curve B that of 20 γ of estrone. Curve C was obtained with mixture of 20 γ each of equilin and estrone. Curve D is the arithmetic sum of Curves A and B and should have coincided with Curve C had the

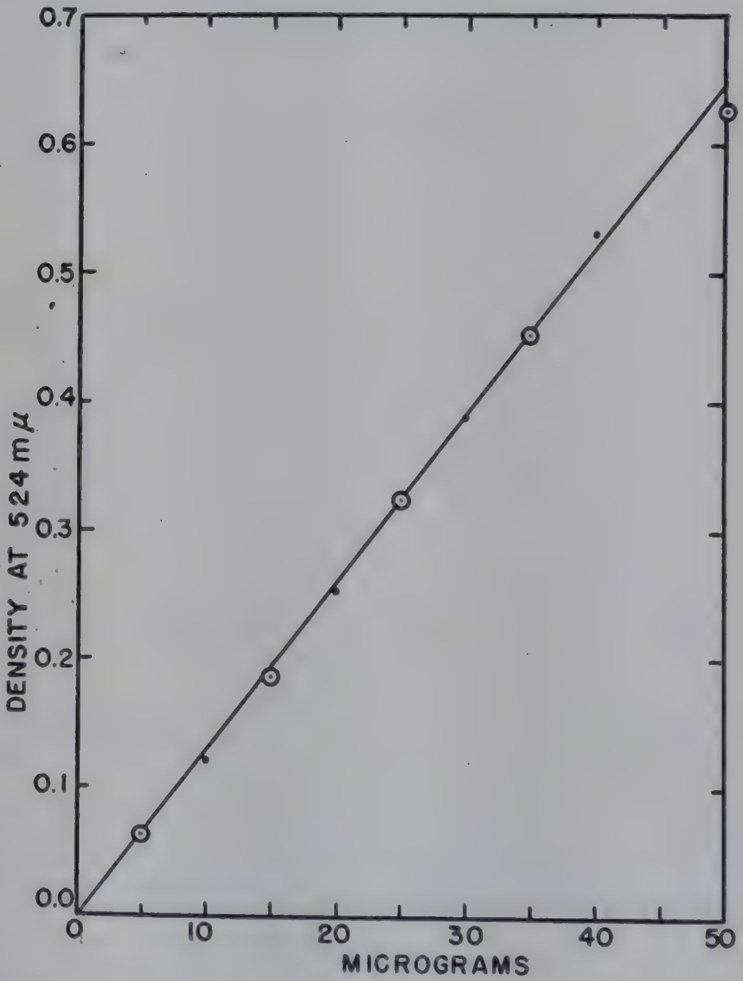

FIG. 6. Applicability of Beer's law to the determination of β-estradiol with the 3 per cent sulfuric acid-20 per cent n-butyl alcohol reagent. The circled points indicate 100 γ of added α-estradiol.

been no interference. There appears to be an inhibition of color throughout the entire spectrum studied. However, the color interference is negligible, as demonstrated by the results on the determination of a series o mixtures shown in Table I.

Identification of Natural Estrogens by Means of Sulfuric Acid Reaction

The spectral absorption curves obtained when the natural estrogens ar heated with 90 per cent sulfuric acid for 12 minutes are sufficiently charac

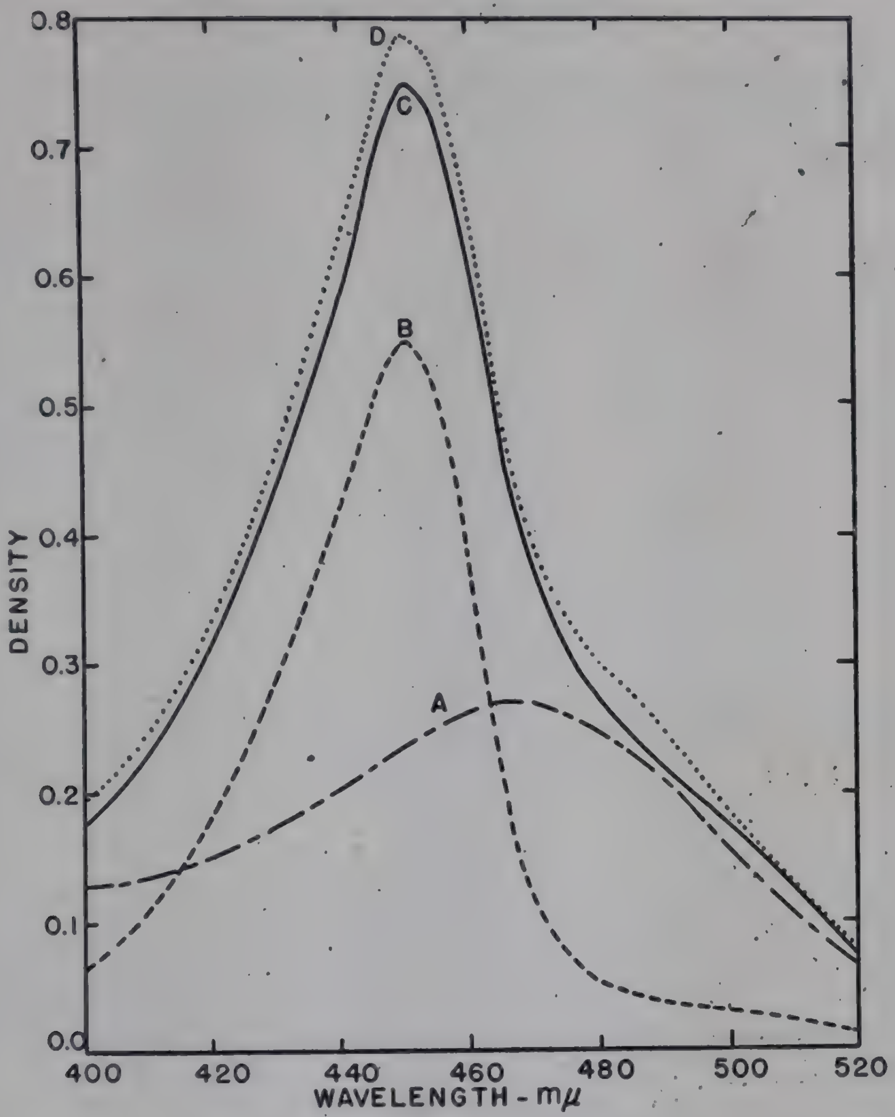

FIG. 7. Absorption spectra of colors produced by heating estrone and equilin separately and in mixtures with 5.0 ml. of 90 per cent sulfuric acid for 5 minutes. Curve A, 20 γ of equilin; Curve B, 20 γ of estrone; Curve C, 20 γ each of estrone and equilin; Curve D, arithmetical sum of values for Curves A and B.

TABLE I

Results Obtained in Determination of Estrone and Equilin in Mixtures in Pure Solution by Heating with 90 Per Cent Sulfuric Acid for 5 Minutes

Density readings at 450 and 480 mμ.

Sample No.	Estrone added	Estrone found	Equilin added	Equilin found
	γ	γ	γ	γ
1	0	1	25	26
2	5	5	40	42
3	5	5	5	5
4	10	11	15	19
5	10	9	30	31
6	15	15	10	10
7	15	15	20	21
8	20	20	0	0
9	20	20	10	11
10	25	26	5	5

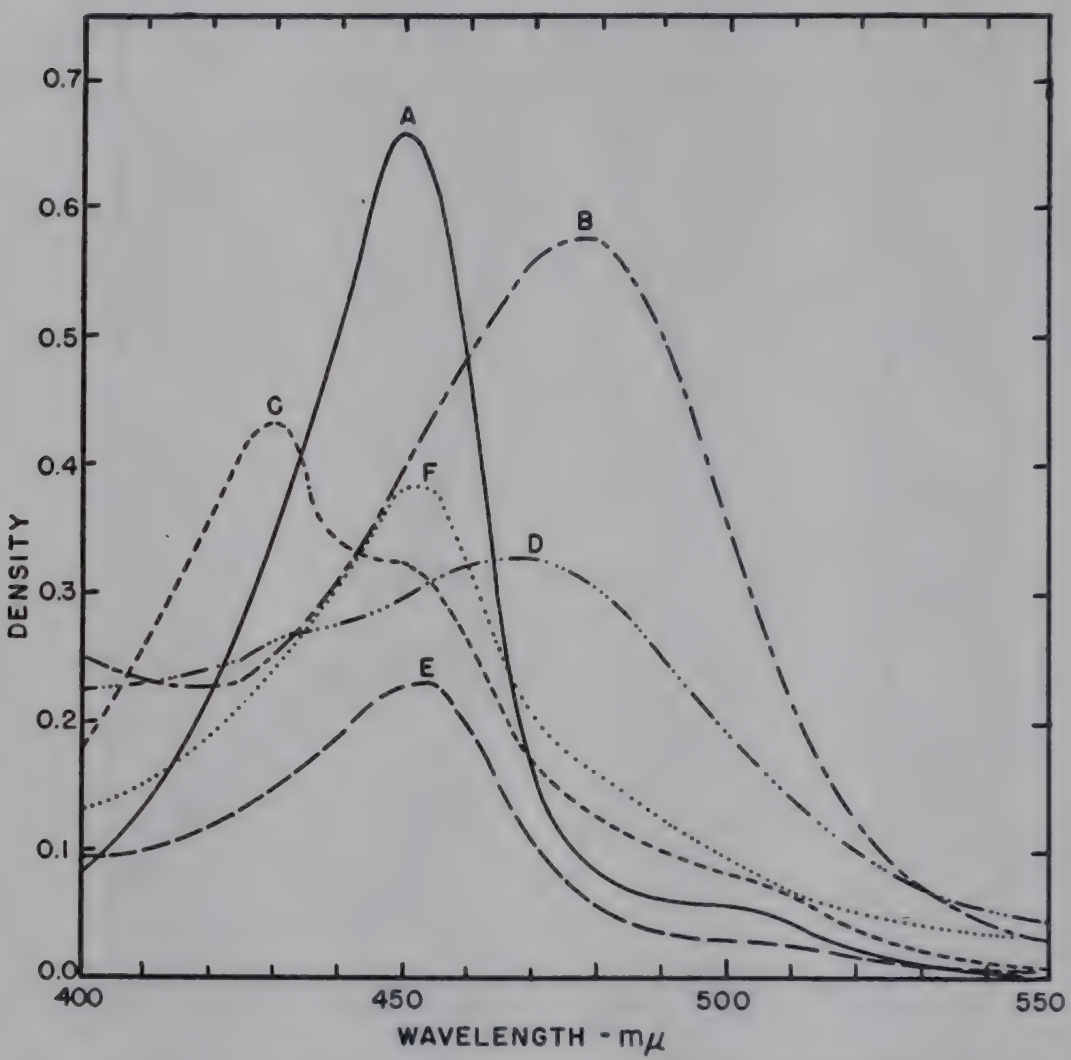

FIG. 8. Spectral absorption curves for six natural estrogens obtained by heating 25 γ of each with 5.0 ml. of 90 per cent sulfuric acid for 12 minutes. Curve A, estrone; Curve B, equilenin; Curve C, α-estradiol; Curve D, equilin; Curve E, β-estradiol; Curve F, estriol.

TABLE II

Identification of Natural Estrogens by Heating with 90 Per Cent Sulfuric Acid for 12 Minutes and Determining Density Values at 430, 450, 480, and 520 mμ

Estrogen	Identification No.	
	$X = \frac{(D^{450})^2}{D^{430} \times D^{480}}$	$Y = \frac{(D^{480})^2}{D^{450} \times D^{520}}$
Estrone	14.92	0.46
Equilin	1.11	3.03
Equilenin	1.09	6.94
α-Estradiol	1.92	1.24
β-Estradiol	5.87	0.86
Estriol	3.80	1.36

teristic for each to serve as a means of identification. Curves so obtained for 25 γ each of estrone, equilin, equilenin, α-estradiol, β-estradiol, and estriol are shown in Fig. 8.

It is not necessary to measure the entire spectral absorption curve in order to obtain an identification. Density readings can be made at 430, 450, 480, and 520 mμ, and from a relationship between these values identification numbers can be calculated. For our purposes, we have calculated an identification number X and an identification number Y. The formulas for their calculation are as follows:

$$X = \frac{(D^{450})^2}{D^{430} \times D^{480}}$$

$$Y = \frac{(D^{480})^2}{D^{450} \times D^{520}}$$

Identification numbers calculated in this manner for the estrogens so far studied are shown in Table II.

DISCUSSION

The methods described here involve a single stage reaction with sulfuric acid. The Kober test, on the other hand, is a two-stage reaction in which the estrogen is first heated with a relatively high concentration of sulfuric acid and then with a low concentration of sulfuric acid. Cohen and Bates (14) have shown that essentially the same results can be obtained in the Kober test without the use of phenol. Apparently, the phenol serves the purpose of a simple diluent in the first stage of the reaction and of quenching the fluorescence in the second stage. As shown here, β-estradiol is the only estrogen so far studied which gives a red color with low concentrations of sulfuric acid. The preliminary heating period with concentrated sulfuric acid used in the Kober test, therefore, must activate the other estrogens in such a way as to cause them to give a red color when heated with the diluted sulfuric acid. This was clearly demonstrated by Cohen and Bates (14) in the case of estrone, when, after a preliminary treatment with concentrated sulfuric acid, a yellow color was obtained if the dilution in the second stage was made with 1:1 sulfuric acid or higher concentrations, and a red color if the dilutions were made with water or dilute concentrations of sulfuric acid. Since β-estradiol is the only compound so far studied which gives a red color with 30 per cent sulfuric acid, it is apparent that it is more reactive and does not need to be activated before it will react. This would explain the results of Carol and Molitor (12) in which β-estradiol reacted with the Kober reagent without a preliminary heating period. It is significant that β-estradiol gives an almost immediate yellow color with 60 per cent sulfuric acid, even at room temperature, while α-estradiol gives little or no color under these conditions. With 73.5 per cent sulfuric acid, however, these observations are reversed.

Just as with the Kober reaction, rigid adherence to manipulative details and concentrations of reagents must be maintained. As illustrated in Fig. 5, the intensity of the absorption varies greatly with the concentration of sulfuric acid. This is particularly of importance in the determination of total estradiols with the 73.5 per cent sulfuric acid. However, in the determination of β-estradiol and the simultaneous determination of estrone and equilin, many of these variables are eliminated by including standards along with the determinations. Since β-estradiol is sensitive to even traces of sulfuric acid, the reaction tubes must be carefully cleaned before each determination. After the tubes are washed exhaustively with tap water, once with a saturated solution of sodium carbonate, and again exhaustively with tap water, they are rinsed out with distilled water, and finally with absolute alcohol.

In addition to being simpler to carry out, the sulfuric acid reaction possesses an advantage over the Kober reaction in that it provides a means of identifying the estrogen in minute amounts. The colors produced in the Kober reaction are in general qualitatively the same. Also, the ultraviolet absorption curves for the estradiols, estrone, and equilin are identical. They can, however, be distinguished by their infra-red absorption (18).

SUMMARY

The behavior of several natural estrogens with various concentrations of sulfuric acid has been studied in detail. The colors produced vary with the estrogen, the time of heating, and the concentration of sulfuric acid The relationship of the sulfuric acid reaction to the Kober reaction has been discussed.

Spectral absorption curves of the colors produced by sulfuric acid with the natural estrogens are sufficiently characteristic to serve as a means o identification of the individual estrogens in minute amounts.

Methods for the quantitative estimation of α-estradiol and β-estradio and of estrone and equilin in binary mixtures in pure solution have been described.

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THE EFFECT OF pH ON THE METABOLISM OF RABBIT BONE MARROW

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In 1924 Warburg and coworkers (1) reported that variation in both the pH and the bicarbonate concentration of the medium affected the anaerobic glycolysis of Flexner-Jobling rat carcinoma *in vitro*. When the bicarbonate level was held constant, an increase in CO_2 tension was accompanied by a fall in the rate of anaerobic glycolysis. At a constant pH, increasing the bicarbonate concentration from 3.1 to 15.5 millimoles per liter was associated with a progressive increase in the rate of anaerobic glycolysis; further increase in bicarbonate concentration produced no significant effect. Craig and Beecher (2) demonstrated that lowering the pH from 7.48 to 7.18 in a bicarbonate-containing medium depressed aerobic glycolysis of rat retina without significant alteration in oxygen consumption. At a constant pH, aerobic glycolysis was increased with increasing CO_2 tensions from 1 to 5 volumes per cent. Similar effects have been reported for the aerobic glycolysis of cat cortex and medulla (3). Warren (4) reported that the presence of bicarbonate resulted in a 20 to 40 per cent increase in the oxygen consumption of rabbit bone marrow.

The work of Summerson, Gilder, and Lee[1] on the effects of pH and bicarbonate on the *in vitro* metabolism of mouse lymphosarcoma prompted parallel studies on normal rabbit bone marrow, which according to Warren (5) exhibits metabolic characteristics approaching those of tumor tissues. An attempt was made to answer the following questions. What are the metabolic characteristics of normal bone marrow at various ranges of pH? Is the metabolism at a given pH influenced by changes in bicarbonate concentration? Can the respiratory metabolism be accounted for in terms of glucose utilization? What fraction of the total aerobic acid production is represented by lactic acid? Can differences be found between the metabolism of bone marrow cells and the cells of lymphosarcoma?

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† Research Fellow of the United States Public Health Service. This work was done in part while associated with the School of Commerce, Accounts and Finance, New York University, New York City.

[1] Summerson, W. H., Gilder, H., and Lee, J. M., in preparation.

EXPERIMENTAL

Methods

The marrow was obtained from the femora and tibiae of male Ne Zealand white rabbits. All animals had been fed *ad libitum*. They wer killed by a blow on the head, bled, and the pencils of marrow from th cracked bones delivered into Ringer's solution at room temperature. Th marrow, free of the larger blood vessels, was pressed with the aid of a smal pestle through an ordinary kitchen sieve into a few ml. of Ringer's solutio in a Petri dish. The resulting tissue suspension was made up to a volum of approximately 16 ml. with Ringer's solution and agitated gently and continuously by a magnetic stirrer to insure uniformity during the subsequen sampling. For the manometric studies, exactly 0.5 ml. of this tissue suspension was pipetted into each manometric vessel. Two 0.5 ml. samples each suspension were taken for the determination of total cell nitrogen.

Examination of stained smears of the marrow, made before and after th preparation of the suspension as described above, gave no evidence excessive cellular destruction. The percentage of myeloid and of erythroi cells per 500 nucleated cells was determined for each animal. The marrow varied from 45 to 65 per cent erythroid in composition. There was no obv ous correlation between metabolic patterns and cellular variation withi this range. No attempts were made to obtain marrows showing wid variation in cellular composition.

The Summerson constant volume differential manometer (6) was use for the manometric measurements. This manometer permits the simu taneous measurement on a single sample of tissue of oxygen consumptio respiratory CO_2 production, and the production of CO_2 due to the tot aerobic acid formation. One may also determine precisely the bicarbona concentration of the medium and the CO_2 tension of the gas phase at bot the beginning and the end of an experiment.[1] From these data, by usi the Henderson-Hasselbalch equation, pH may be determined at the begi ning and end of an experiment, and by interpolation for any time duri the experimental period. These calculated values agree within 0.03 u of pH with those measured in a Beckman pH meter.[2] We have chosen report as most representative the pH at the mid-time of the experiment run; *i.e.*, the pH of the medium 60 minutes after the beginning of t experiment.

The total volume of the manometer vessels was approximately 18 Each vessel contained exactly 1.0 ml. of Ringer-bicarbonate-glucose sol tion added to the 0.5 ml. of marrow suspension, with 0.2 ml. of 2.5 N H in the side bulb. The composition of the Ringer's solution was sodi

[2] Personal communication from Dr. W. H. Summerson.

chloride 8.65 gm. per liter, potassium chloride 0.23 gm. per liter, calcium chloride 0.24 gm. per liter. The concentration of glucose was approximately 500 mg. per cent. According to the experimental pH desired, varying amounts of 0.15 M sodium bicarbonate were used in the preparation of the Ringer-bicarbonate-glucose solution. To bring this solution to the approximate pH desired, it was equilibrated for 20 minutes with the appropriate O_2-CO_2 gas mixture before being placed in the vessels. The gas mixtures varied from 80 per cent O_2-20 per cent CO_2 to 97.5 per cent O_2-2.5 per cent CO_2.

The experimental temperature was 37.5°, and the shaking rate approximately 120 strokes per minute. Prior to an experiment the appropriate gas mixture necessary to establish approximately the desired pH was passed through the vessels for a 20 minute equilibration period with the vessels in the thermostat at 37.5°. The exact pH was determined experimentally, as described above.[1] An equal rate of gas flow was maintained through all the vessels during the preliminary gassing period. 5 minutes were then allowed for equilibration at atmospheric pressure and a final 5 minutes for temperature equilibration. At zero time, acid was tipped into the control vessels. All aerobic experiments were 120 minutes in duration. Upon the completion of the experiment, acid was tipped into the experimental vessels.

The method of determining anaerobic glycolysis was essentially that outlined by Umbreit (7). The gas mixture was composed of 95 per cent N_2-5 per cent CO_2. Variations in pH were obtained by varying the molarity of the bicarbonate in the medium. Anaerobiosis was established by passing this gas mixture through the vessels for a 30 minute equilibration period. Anaerobic experiments were of 60 minutes duration.

To the total contents of each vessel was added 0.5 ml. of 10 per cent sodium tungstate for the precipitation of protein, at a final volume of 25 ml. This was filtered and the filtrate analyzed for glucose and lactic acid. Glucose was determined by the method of Benedict (8) in the Klett-Summerson photoelectric colorimeter. Lactic acid was determined according to the method of Barker and Summerson (9). The analytical accuracy was controlled for each series of experiments by the ability to recover in a separate sample known additions of glucose and lactic acid. Recovery errors averaged less than 3 per cent. With the manometric procedure used here, glucose utilization or lactic acid formation is established by the difference between analytical results in control and experimental vessels.

In this paper oxygen consumption is symbolized by the conventional Q_{O_2} and is defined as the number of c.mm. of O_2 consumed per mg. of cell protein per hour. Q_{CO_2}, $Q_G^{O_2}$, and $Q_G^{N_2}$ are expressed in corresponding units, where Q_{CO_2} represents respiratory CO_2, and $Q_G^{O_2}$ or $Q_G^{N_2}$ represents aerobic or anaerobic acid production, in terms of CO_2 liberated from bicarbonate.

$Q_{\text{Gluc.}}$ and Q_{LA} (glucose and lactic acid, respectively) are expressed in micro grams per mg. of cell protein per hour. Total cell nitrogen was determine by the Kjeldahl method and was converted into total cell protein by th customary factor of 6.25. If desired, the Q values as given here may b converted into terms of "fat-free dry weight" (10) by multiplying by th factor 0.91.

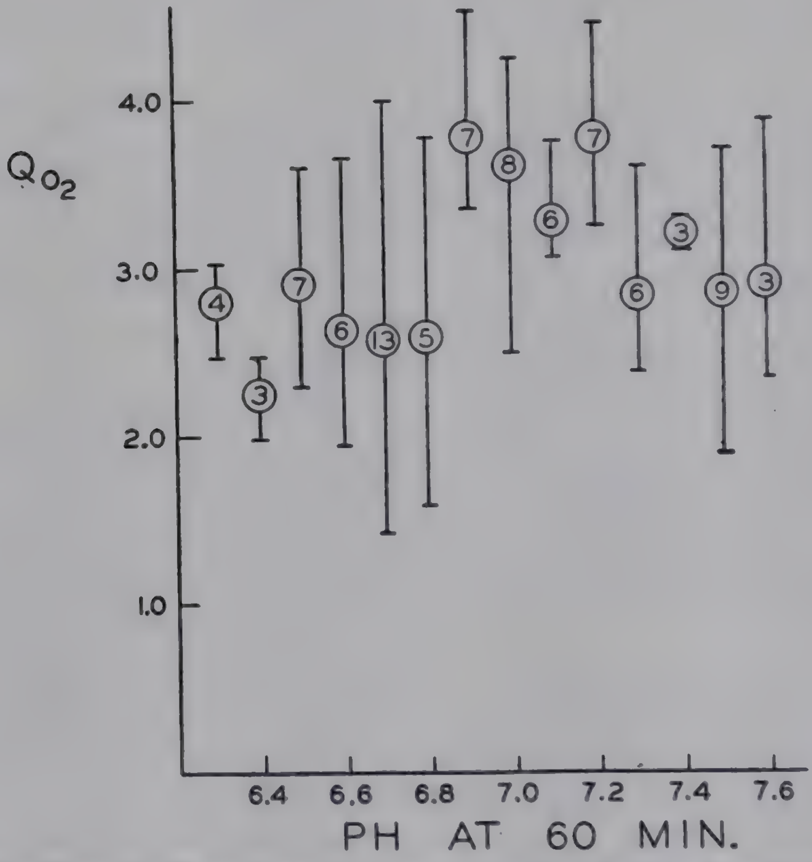

FIG. 1. The effect of pH on the oxygen consumption of normal rabbit bone marro cells. The average Q_{O_2} at each pH range is represented by the position of the circl the number within the circle representing the number of experiments performe The spread of the vertical lines represents the spread of the individual determinatio within each range of pH.

Results

87 aerobic experiments covering a pH range from 6.3 to 7.7 have be performed. To simplify the presentation of the data this range was su divided into parts, each having a span of one-tenth of a pH unit, and on th basis the experimental results have been grouped. In the figures, t averages of the Q values of each group are represented by circles. T numbers within the circles indicate the number of experiments performe The range of variation from the arithmetical average of each group is repr sented by the span of the vertical lines.

The relation of oxygen consumption to the calculated pH of the mediu at the mid-time of the experimental period is presented in Fig. 1. Oxyg consumption was greatest in the pH range 6.9 to 7.3. For this range the average oxygen consumption was 3.6 c.mm. per mg. of cell protein per hour. The spread of Q_{O_2} values from the average was large in each range of pH studied and the differences between the averages were small over the entire pH range.

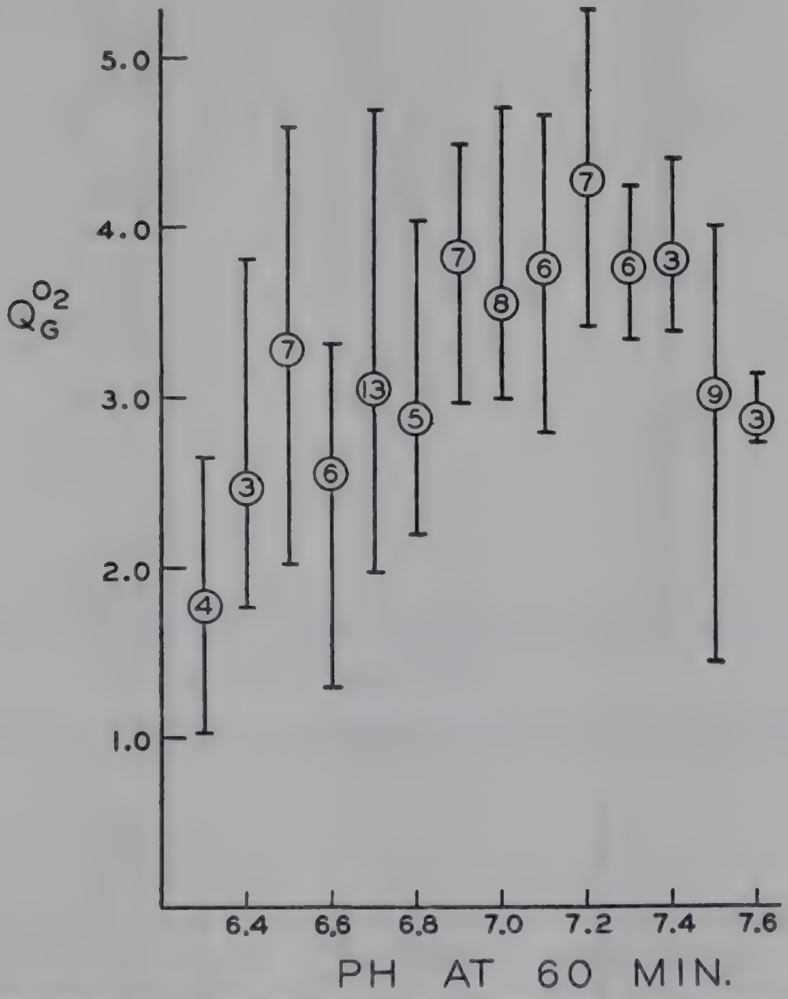

FIG. 2. The effect of pH on the aerobic acid production of normal rabbit marrow.

From pH 6.3 to 7.3 the total aerobic acid production increased with increasing pH in a substantially linear fashion. This relation of $Q_G^{O_2}$ to pH is shown in Fig. 2. Aerobic glycolysis was greatest at pH 7.2, the average $Q_G^{O_2}$ being 4.3; it was smallest at pH 6.3, the average $Q_G^{O_2}$ being 1.8. Above pH 7.3 the glycolytic rate diminished with increasing pH to an average of 2.9 at pH 7.6.

The lactic acid production of aerobic glycolysis is related to pH in Fig. 3. The averages for the Q_{LA} of each pH group fall into a pattern which approximates that shown in Fig. 2. At pH 6.3 Q_{LA} averaged 7.6; at pH 7.1 it averaged 15.9. In pH ranges above 7.1, lactic acid production was diminished with increasing pH and Q_{LA} averaged 8.9 at pH 7.6. The ratio between total acid and lactic acid production for each pH grouping is shown

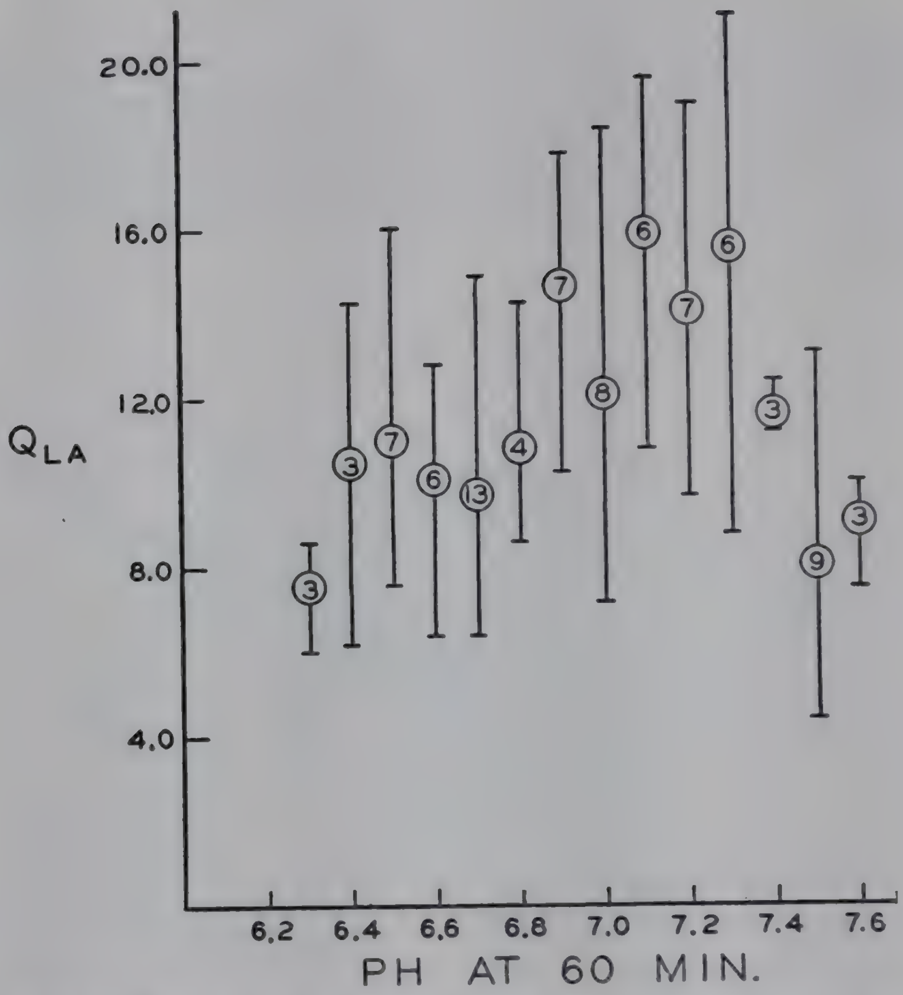

Fig. 3. The effect of pH on the production of lactic acid by rabbit marrow

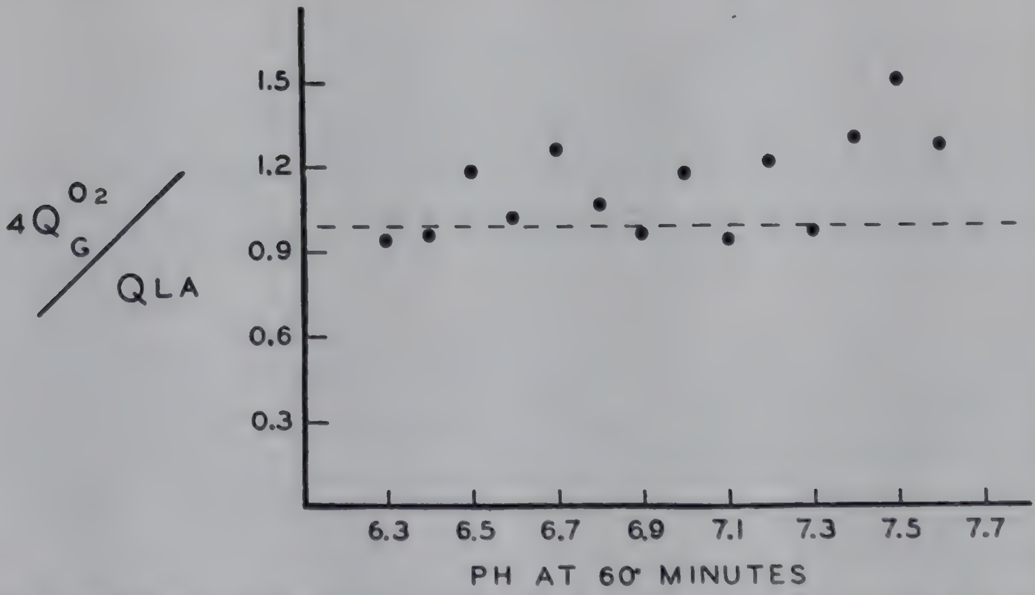

Fig. 4. The effect of pH on the ratio of total acid production to lactic acid forr
tion in rabbit marrow.

in Fig. 4. In Fig. 4, $Q_G^{O_2}$ has been multiplied by 4 to convert it into ter
equivalent to Q_{LA}. When total acid formation exceeds lactic acid prod

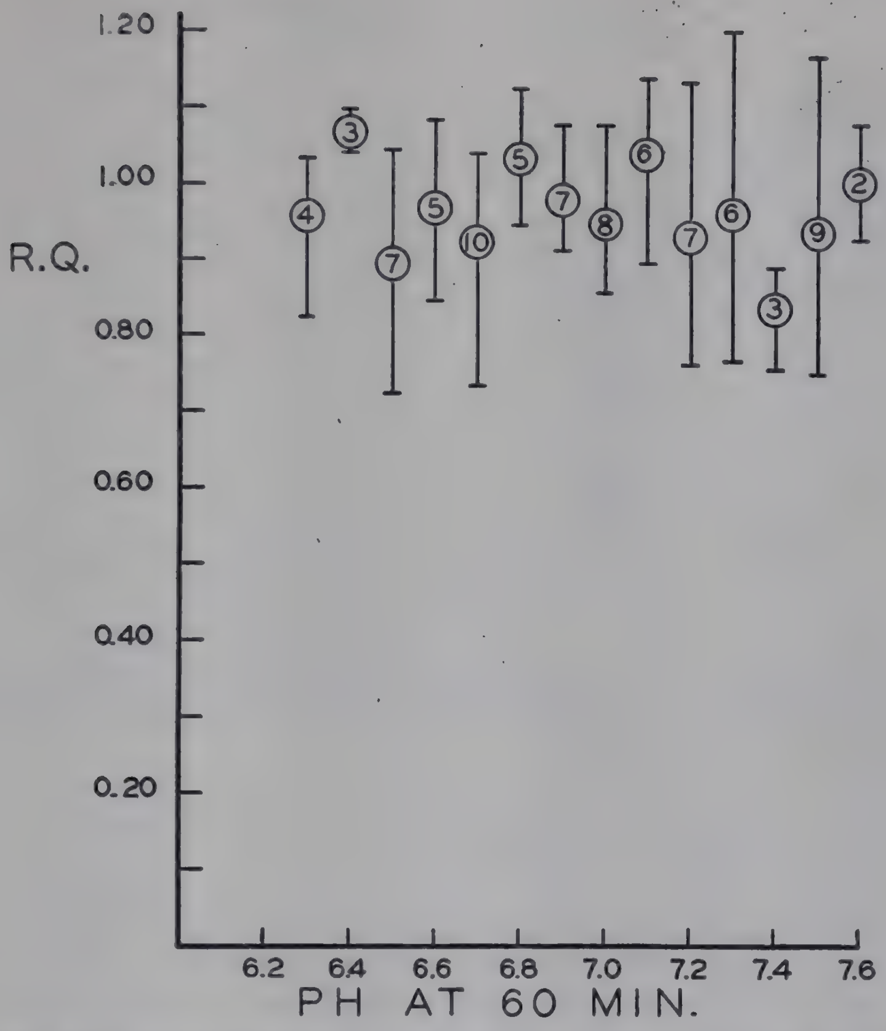

Fig. 5. The effect of pH on the respiratory quotient in normal rabbit bone marrow cells.

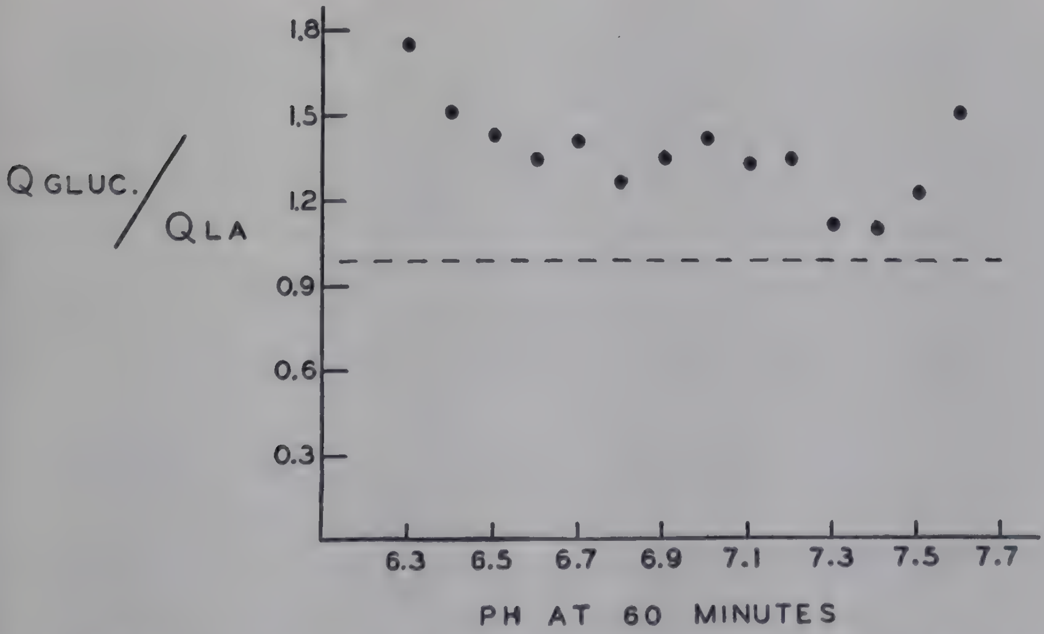

Fig. 6. The effect of pH on the ratio of glucose consumption to lactic acid production in rabbit marrow.

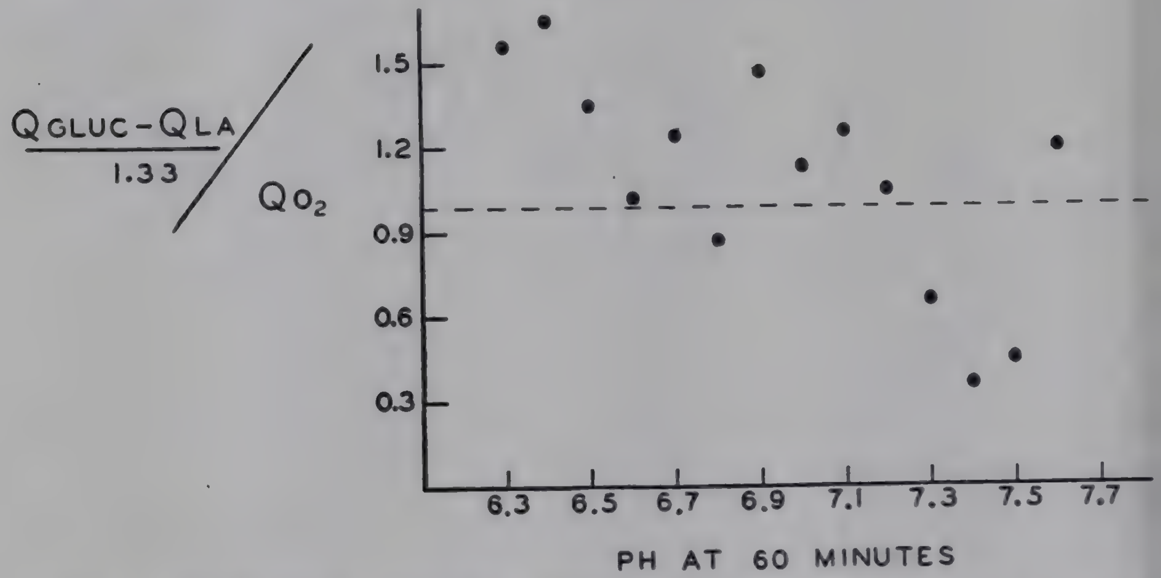

Fig. 7. The ordinate of this graph represents the ratio of the oxygen equivalen of the glucose utilized in excess of lactic acid produced to the total oxygen consumption. This ratio is related to pH.

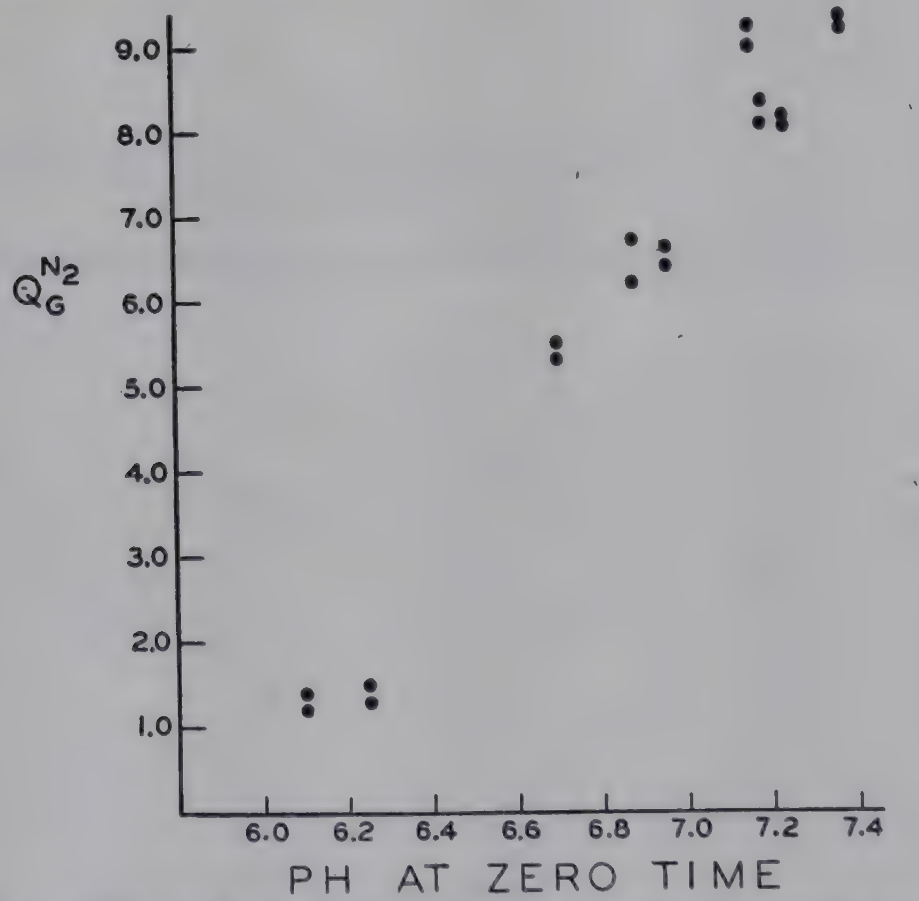

Fig. 8. The effect of pH on the anaerobic acid production of normal rabbit ma row.

tion, the ratio is greater than 1. Such a ratio exists in nine of the fourte groups. The average lactic acid production of all experiments was 88 p cent of the average for total acid formation.

pH exerted no effect on R.Q. This is shown in Fig. 5. For all experiments the R.Q. averaged 0.95. Such a value directs attention to the glucose utilization by the marrow. At pH 7.1, $Q_{\mathrm{Gluc.}}$ averaged 21.5. It was greatest at pH 6.9, averaging 22.2. At pH 6.3 and 7.6, $Q_{\mathrm{Gluc.}}$ was low, averaging 13.5 and 13.6, respectively. As defined in this paper, $Q_{\mathrm{Gluc.}}$ and Q_{LA} may be compared directly. Their ratio is related to pH in Fig. 6. A value greater than 1 in Fig. 6 exists when glucose utilization exceeds lactic acid production. As may be seen in Fig. 6, all values are greater than 1. The glucose utilization in excess of lactic acid formation is theoretically available for oxidation. The ratio of the oxygen equivalent of this excess glucose to the Q_{O_2} is related to pH in Fig. 7. Micrograms of glucose have been converted to equivalent microliters of oxygen by dividing the value in micrograms by 1.33. In Fig. 7, a ratio greater than unity indicates that more glucose is utilized than can be accounted for by both lactic acid production and oxygen consumption. In ten of the fourteen groups the ratio is greater than 1.

The manometric results of eighteen experiments on the anaerobic glycolysis of normal rabbit marrow cells are presented in Fig. 8. The marrow of three animals was used. It is apparent that the $Q_{\mathrm{G}}^{\mathrm{N}_2}$ is affected in a linear fashion by changes in pH, averaging 9.3 at pH 7.4 and 1.3 at pH 6.2. Lactic acid production was also linearly affected by pH and accounted for the total anaerobic acid formation. Glucose utilization paralleled lactate production in approximately a 1:1 ratio throughout the pH range studied.

DISCUSSION

We feel that it is important to stress precise knowledge of the bicarbonate concentration in the medium under experimental conditions. In order to draw conclusions regarding bicarbonate or pH effect on glycolysis or other aspects of cell metabolism, it is not sufficient merely to know the molarity of the bicarbonate in the medium prior to the equilibration period. This is illustrated by one typical set of experiments. Prior to the introduction of tissue, the Ringer-bicarbonate-glucose solutions contained 5.7, 11.2, and 22.4 mM of bicarbonate per liter, respectively. At the start of the experiment, after the preliminary equilibration, the bicarbonate concentrations for the same solutions were 3.5, 7.4, and 13.2 mM per liter. The breakdown of bicarbonate during the equilibration period is large, variable, and consequently not accurately predictable. Not only does this influence the calculation of pH but also, when one uses small quantities of bicarbonate in the medium, a rapidly glycolyzing tissue may decompose all of the bicarbonate either during equilibration or prior to the completion of the experimental period. Consequently, the interpretation of investigations reporting the effects on cell metabolism of either $p\mathrm{CO}_2$ or of bicarbonate may be subject to an unpredictable error when these quantities are not measured under conditions existing during the experimental period.

In the experiments here reported the average Q values for normal rabbit marrow in a bicarbonate-containing medium at pH approximating 7.4 were Q_{O_2}, 3.2; $Q_G^{O_2}$, 3.9; Q_{LA}, 11.8; and $Q_{Gluc.}$, 13.4. These values for respiratory metabolism are very close to those previously reported in which the medium was Ringer-phosphate solution (5, 10, 11). Under experimental conditions different from those of the present study, Warren (4) reported that the respiration of rabbit bone marrow was approximately 40 per cent greater in Ringer-bicarbonate than in Ringer-phosphate solution. We are aware of no experiments comparable to those reported here on the effect of pH change on the *in vitro* respiration and glycolysis of bone marrow. Similar studies have been made with rat retina (2), cat cortex (3), and mouse lymphosarcoma (12).

TABLE I

Partial Tabulation of Bicarbonate Concentration, pCO₂, and Aerobic Glycolysis of Marrow in Range of pH 6.70 to 6.79

(HCO_3^-), 0 time	CO_2, 0 time	$Q_G^{O_2}$	(HCO_3^-), 0 time	CO^2, 0 time	$Q_G^{O_2}$
mM per l.	*vol. per cent*		*mM per l.*	*vol. per cent*	
5.9	4.3	3.2	9.6	7.4	4.1
5.9	4.6	2.0	9.9	9.1	2.0
6.2	4.9	4.5	10.9	9.2	3.2
6.3	4.6	2.4	11.1	8.8	3.5
8.4	7.3	2.7	11.2	9.7	4.7
9.1	7.5	2.2	11.9	9.9	2.2

In each range of pH studied, the total aerobic acid production was greate than the lactic acid formation. No attempts were made to identify othe than lactic acid. In ten of the fourteen groups into which the experiment were divided on the basis of pH, more glucose was utilized than could b accounted for by both lactic acid formation and oxygen consumption. I the analyses for glucose are dependable, this fact implies glycogenesis o the part of the marrow or the formation of intermediate carbohydrat metabolites. On the basis of incompleted studies, we doubt that significan glycogenesis occurs. No analyses were made for carbohydrate intermedi aries. The work of Goldinger, Lipton, and Barron (11) indicates that i marrow glucose is metabolized by way of the Krebs' tricarboxylic acid cycl and that there is a small synthesis of citric acid.

In view of the current interest in the utilization of carbonic acid in cellula metabolism (13), the question arises as to whether the bicarbonate concen tration or the pCO_2 influenced results *per se*. Various concentrations bicarbonate and carbon dioxide yielding the same pH were studied. Within any small range of pH, the glycolytic rates showed no recognizable trends which could be attributed to increasing molarity of bicarbonate. Table I presents a partial tabulation of experiments performed at pH 6.70 to 6.79.

Comparison of our data for normal rabbit marrow cells with those of Summerson, Gilder, and Lee (12) for mouse lymphosarcoma shows certain differences. Aerobic glycolysis of lymphosarcoma seems to be more sensitive to changes in pH than does that of marrow. This difference appears to be quantitative. In lymphosarcoma, aerobic glucose utilization can be entirely accounted for on the basis of lactic acid formation. Such is not the case with marrow. The R.Q. of marrow was found to be 0.95, whereas that of lymphosarcoma was lower, averaging 0.83. In general lymphosarcoma is a more actively metabolizing tissue than marrow. This is not the comparison of a normal tissue with its malignant counterpart. Rather it is a comparison of two distinct tissues, studied by identical experimental techniques, in each of which the pH of the medium markedly affected the rate of glycolysis but had slight effect on the respiratory rate. Therefore, we feel that any attempt to relate glycolysis and respiration in a quantitative way as a means of characterizing a tissue metabolically is open to question when the effect of pH has not been determined.

Grateful acknowledgment is made to Dr. W. H. Summerson for his interest and help in connection with the material presented here.

SUMMARY

1. The *in vitro* metabolism of rabbit bone marrow cells in Ringer-bicarbonate-glucose medium has been studied with the Summerson differential manometer. 87 experiments were performed under aerobic conditions, eighteen anaerobically. Analyses for glucose utilization and lactic acid production by the tissue have been compared with the manometric data. All metabolic rates have been correlated with the pH of the medium, as determined during the experimental period.

2. Aerobic metabolic rates were maximum at pH 7.2. Both aerobic and anaerobic glycolysis were depressed markedly and in linear fashion by depression of the pH of the medium from 7.2 to 6.3. The effect of change of pH on the oxygen consumption and R.Q. was small, the R.Q. averaging 0.95. Total aerobic acid formation was greater than aerobic lactic acid production. Aerobic glucose utilization was at least sufficient to account for both lactic acid production and oxygen consumption.

3. Since pH markedly affects the glycolytic rate but has small effect on oxygen consumption, any ratio relating glycolysis to respiration in rabbit bone marrow is variable. Such is also the case with mouse lymphosarcoma

and probably other tissues as well. Hence, the significance of such a rat in characterizing the metabolism of a tissue is questioned unless the infl ence of pH is taken into account.

4. The breakdown of bicarbonate in the medium during the prelimina equilibration period was large and variable. Consequently, the validity experiments reporting the effects on cell metabolism of either bicarbona or pCO_2 is also questioned when these quantities have not been measur under conditions existing during the experimental period.

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THE β-CARBOXYLASES OF PLANTS

I. SOME PROPERTIES OF OXALACETIC CARBOXYLASE AND ITS QUANTITATIVE ASSAY*

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The ability of the higher plants to utilize carbon dioxide for the synthesis of organic compounds has long been recognized, whereas the more limited ability of heterotrophic organisms to cause "CO_2 fixation" by carbon to carbon linkage has been established only within recent years (1). Nevertheless, there is more detailed information available at present about the mechanisms of CO_2 fixation in bacteria and animal tissues than about the mechanisms of CO_2 assimilation in plants. This state of affairs is undoubtedly due to the relatively much greater complexity of the latter processes, since photosynthesis results eventually in complete synthesis, from carbon dioxide, of all the carbon chains of the plant organism.

On the initiation of a study of CO_2 fixation in higher plants several years ago (2), we thought it worth while to simplify the problem by eliminating the process of utilization of light energy, and proceeded on the assumption that "dark fixations" of CO_2 in plant tissues could occur by paths similar to those already established in heterotrophs. In particular, the possible occurrence of enzymes catalyzing the reversible decarboxylations of oxalacetic acid (OAA) and oxalosuccinic acid (OSA) was investigated. Such enzymes had been demonstrated and studied in bacteria by Krampitz and Werkman (3) and in animal tissues by Evans and his collaborators (4, 5), Utter and Wood (6), and by Ochoa and his collaborators (7–9).

As a result of these studies, it has been found that crude protein preparations from parsley root can catalyze reactions (*a*) through (*d*) (10, 11).[1]

(*a*) $$\text{Oxalacetate} \rightleftarrows CO_2 + \text{pyruvate}$$

(*b*) $$\text{Oxalacetate} + TPN_{\text{red.}} \rightleftarrows \text{malate} + TPN_{\text{ox.}}$$

(*c*) $$\text{Oxalosuccinate} \rightarrow CO_2 + \alpha\text{-ketoglutarate}$$

(*d*) $$\text{Oxalosuccinate} + TPN_{\text{red.}} \rightleftarrows \text{isocitrate} + TPN_{\text{ox.}}$$

The present communication will describe some of the properties of this enzyme preparation, particularly in relation to OAA carboxylase, *i.e.*, the

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* Aided in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] TPN = triphosphopyridine nucleotide; DPN = diphosphopyridine nucleotide; ATP = adenosine triphosphate.

enzyme catalyzing reaction (*a*). A number of observations on β-carboxylases from other plant sources will be included. The distribution of thes enzymes will be described in another communication (12).

Methods and Material

Standard Warburg manometric techniques were used throughout. Spectrophotometric measurements were made with a Beckman quartz spectrophotometer. Dry weight determinations were made by drying at 110° fo 24 hours, and were corrected for the known buffer content of the samples The *l*-malic acid was a recrystallized commercial preparation. Diphosphothiamine was generously supplied by Merck and Company. Diphosphopyridine nucleotide was a commercial sample of 40 per cent purit purchased from the Schwarz Laboratories. The preparation of other substrates and cofactors and the procedures employed in the exchange experiments with $C^{14}O_2$ have been described elsewhere (5).

Preparation of Oxalacetic Carboxylase from Parsley Root—Parsley root were peeled, ground to a pulp in a meat grinder, and the juice pressed ou with a fruit press. The peeling could be omitted without damage to th enzyme activity, but in this case a brown pigment contaminated the fina preparation. The amount of juice obtained varied with different batche of roots, but no advantage was gained by adding water or buffer in severa attempts to extract additional activity from the pulp.

The juice expressed from freshly ground parsley roots is a cloudy, yello liquid, very rich in pyruvic carboxylase. Quantitative assay of the oxa acetic carboxylase activity at this stage is therefore not feasible. The fres juice was treated immediately with 0.05 volume of 3 M acetate buffer, pH 5.0, and allowed to stand at 4° for from 2 to 12 hours. The precipitate wa centrifuged, and the supernatant was dialyzed for 48 hours in the col against 0.025 M phosphate buffer, pH 7.4. The precipitate which forme was centrifuged, and the rates of decarboxylation of oxalacetic and pyruv acids catalyzed by the supernatant were determined.

If the pyruvic carboxylase activity was sufficiently low to allow a dete mination of the first order rate constant of the oxalacetic enzyme, as d scribed later, the preparation was lyophilized at this point. Otherwise, tl treatment with acetate buffer, centrifugation, and dialysis were repeate At times the acetate treatment was repeated twice before lyophilizin This procedure is not recommended unless the pyruvic carboxylase activit is exceptionally resistant to inactivation, since the acetate treatment alwa leads to some loss of OAA carboxylase activity. It was always possible remove the last traces of pyruvic carboxylase by prolonged dialysis (3 to days) of a concentrated solution made up from the lyophilized powder.

Besides destroying pyruvic carboxylase, the acetate treatment yields enzyme solution which can be cleared of turbidity by centrifugation. Repeated acetate treatments may be necessary to achieve this purpose. By a combination of acetate treatment, dialysis, and centrifugation, we have been able to prepare perfectly clear enzyme solutions free from pyruvic carboxylase from almost every batch of roots tested. About one trial in ten was unsuccessful, leading to a product containing so little activity that it was discarded.

This procedure could be applied to parsnip as well as to parsley root, with virtually identical results; *i.e.*, the final preparations obtained appeared to be indistinguishable. Attempts to apply it to carrot roots and to radishes gave preparations in which the presence of OAA carboxylase could be demonstrated, but yields were low. Subsequent work by Dr. J. Ceithaml has indicated that direct precipitation of plant press-juices with ammonium sulfate is superior to the procedure described above. All experiments on OAA carboxylase reported in this paper were carried out, however, on enzyme solutions prepared in the manner described.

Quantitative Assay of Oxalacetic Carboxylase—For the quantitative assay of the amount of OAA carboxylase present in a given preparation, the rate of evolution of carbon dioxide from added OAA is measured manometrically. The reaction medium contains 0.1 M acetate buffer, pH 5.0, 0.01 M $MnCl_2$, and 1 mg. of OAA. The reaction is run in air at 30°. Reaction (*a*) proceeds completely to the right under these circumstances, since there is little retention of carbon dioxide in the medium. All β-carboxylases obtained from plant sources have been found to exhibit first order kinetics with respect to substrate when the reaction is followed under these conditions.

We have adopted the procedure of measuring equal amounts of enzyme into two Warburg vessels and inactivating one sample by holding the vessel in a boiling water bath for 1 minute. Then acetate and $MnCl_2$ are added in amounts to make a final concentration of 0.1 M acetate and 0.01 M $MnCl_2$ and the volume is made up to 1.9 ml. In the side arm of each vessel is placed 0.1 ml. of H_2O containing about 1 mg. of OAA which is made up immediately before use by dissolving about 10 mg. of OAA in 1 ml. of H_2O. The vessels are equilibrated for 10 minutes and the reaction started by tipping. A reading is taken at 1 or 2 minutes and readings are spaced thereafter at suitable intervals, depending on the speed of the reaction. It is necessary to time readings accurately, and no more than six vessels are therefore run at one time. The reaction stops completely when the CO_2 evolved is equivalent to the OAA added. As a check on this figure, a vessel is always made up with acetate buffer, 0.03 M $NiSO_4$, and 0.1 ml. of the same OAA solution that was used in the activity determination. Oxalacetate is completely decarboxylated in 15 minutes in this system, and the amount of oxalacetate added at the time of tipping can then readily be determined. From the microliters of CO_2 evolved and the amount of OA added, the amount of OAA present at any time may be calculated. The lo of the OAA concentration plotted against time gives a straight line with slope proportional to the velocity constant, k, of the reaction. The con

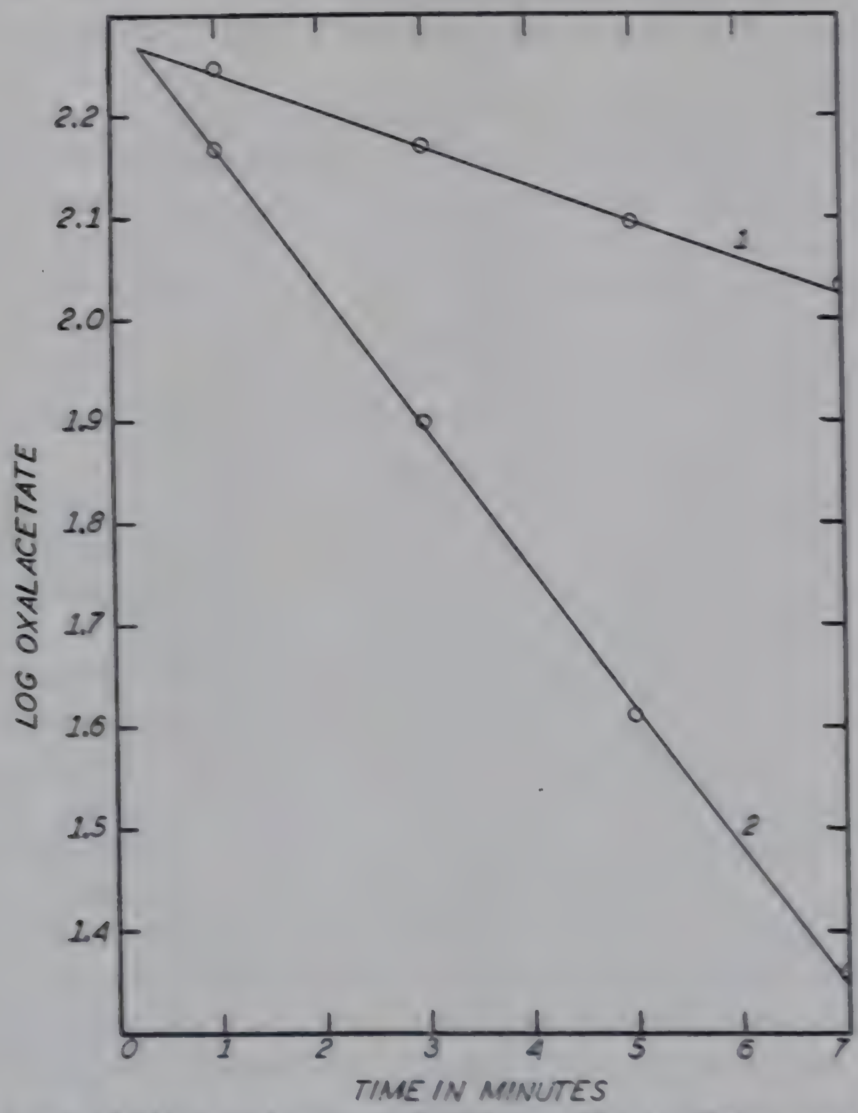

FIG. 1. Assay of plant oxalacetic carboxylase. The conditions are those of t standard test system. 0.01 M $MnCl_2$, 0.1 M acetate buffer, pH 5.0; 196 μl. of OA added at time of tipping. Amount parsley root enzyme, 0.3 ml. of a solution contai ing 10 mg. of dry weight. Curve 1, enzyme heat-inactivated; Curve 2, enzyme n heat-inactivated. Slope of Curve 1, (Δ log OAA)/(Δ minutes) = 0.036; slope Curve 2, 0.136; activity, k per ml. = (2.3(0.136 − 0.036)/0.3) = 0.77; net dry weig = 10 mg. per ml.; activity, k per mg. = 0.077.

stant is defined by the equation

$$k = \frac{2.30}{t_2 - t_1} \log \frac{\text{OAA}_1}{\text{OAA}_2}$$

in which OAA_1 and OAA_2 are OAA concentrations at times t_1 and t_2 respe tively. Results of a typical assay are shown in Fig. 1.

The difference between the first order rate constant obtained in the presence of the unheated enzyme and the first order rate constant obtained in the presence of heat-inactivated enzyme (or in the absence of enzyme) is directly proportional to the amount of enzyme present, as shown in Fig. 2. In most instances, heat-inactivated enzyme (held in a boiling water bath 1 minute) has no effect on the velocity of the decarboxylation, which is that due to the divalent cation alone. This value is referred to as the blank. Occasionally, however, crude preparations will affect the blank after heat inactivation, and we have therefore usually determined blanks in the presence of heat-inactivated enzyme.

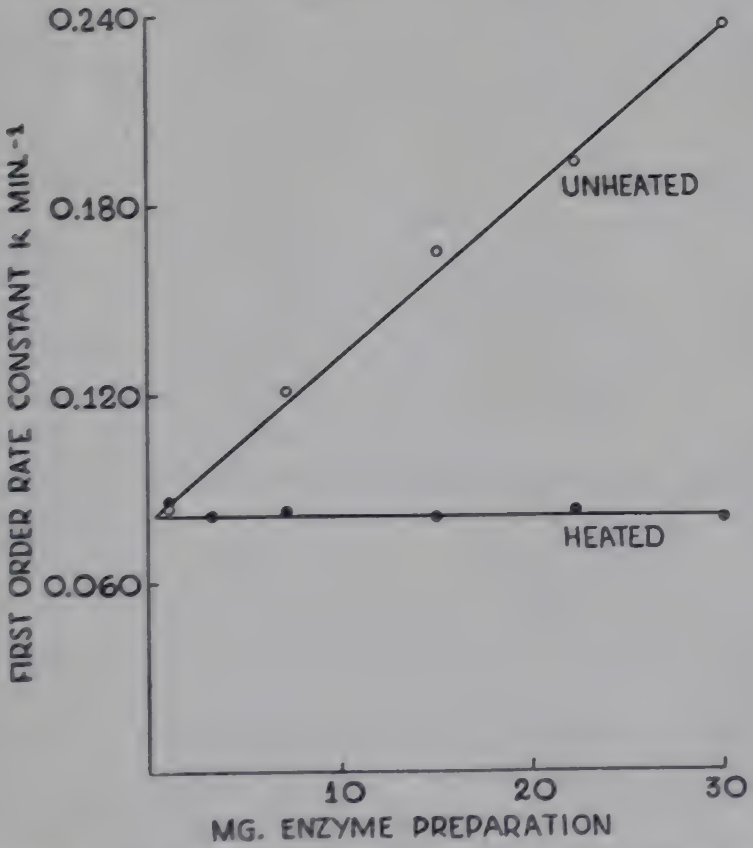

FIG. 2. Proportionality of first order rate constant to quantity of enzyme. The conditions are those of the standard test system. In this case mg. of enzyme preparation refers to the total weight of lyophylized powder.

The Mn^{++} concentration of the test system was chosen to give maximum enzyme activity (13). The pH and the concentration of buffer were rather arbitrarily selected. The enzyme activity decreases as the buffer concentration increases. Thus, at acetate concentrations of 0.05, 0.25, and 1.25 M, the relative enzyme activities were 14, 5, and 1, respectively. This seems to be a non-specific salt effect, since addition of sodium chloride gives similar effects at similar concentrations. It is necessary, however, to have sufficient buffer present to prevent the pH change associated with the removal of a carboxyl group during the course of the reaction. The optimum pH of the enzyme has not been determined, but it lies above pH 5.0. Thus the relative activities of an enzyme tested in acetate buffer at pH 4.4, 5.0, and 5.5 were found to be 1, 4, and 7 respectively. The increased activity observed above pH 5.0 is balanced by the decreased activity associated with the need for higher buffer concentrations at the latter pH. Therefore the choice of buffer concentrations and pH is a compromise selected for feasibility. Activity measurements made as described are reproducible within 10 per cent and directly proportional to the enzyme concentration, but do not represent maximum or optimum activity.

Although assays were routinely run in air, attention is called to the need for precautions due to possible oxygen consumption (5). If the reaction is run to completion, the CO_2 evolved should be equal to that in the $NiSO_4$-catalyzed decarboxylation which is always run simultaneously. This agreement is a check on the absence of any oxygen consumption. We have virtually never observed more than 10 μl. of O_2 consumption per hour when assaying plant oxalacetic carboxylases prepared as described.

Properties of Oxalacetic Carboxylase Preparations—The enzyme preparation described contains a number of enzyme activities in addition to oxalacetic carboxylase. Thus the presence of oxalosuccinic carboxylase and of isocitric and malic dehydrogenases active with TPN has already been reported (10, 11). (Further details regarding the tricarboxylic acid system will appear in a separate paper (14).) It is not possible at present to say to what extent the two β-carboxylases are associated, or to what extent the respective dehydrogenases are necessarily present with the carboxylases. The dehydrogenases cannot be demonstrated readily in all preparations. At present it appears, however, that this may be due to the presence of an enzyme or enzymes (in variable amounts in different preparations) which destroy TPN.[2] Malic dehydrogenase active with DPN has been found either absent or present in only small amounts. Here again, however, the possible presence of DPN-destroying factors may influence the results. Lactic dehydrogenase, fumarase, and aconitase have never been observed in any of the preparations tested.

The OAA carboxylase in the preparations described is quite stable at neutral pH and can be kept at 4° for at least a week without loss in activity. It can be dialyzed at pH 7 to 8 against dilute phosphate or veronal buffer, likewise with no loss in activity. Though phosphate was usually employed for the dialyses, it is not necessary for enzyme action. Small and variable losses in activity accompanied dialysis against distilled water, but this is probably associated with the acid pH of the water. There is no evidence for the presence of a dialyzable cofactor other than divalent cations.

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[2] We are indebted to Mr. Eric Conn of this department for information regarding the action of the plant enzyme preparations on TPN.

The activity of the carboxylase is not influenced by TPN, DPN, diphosphothiamine, or ATP. Considerable inhibition is produced by *l*-malic acid.

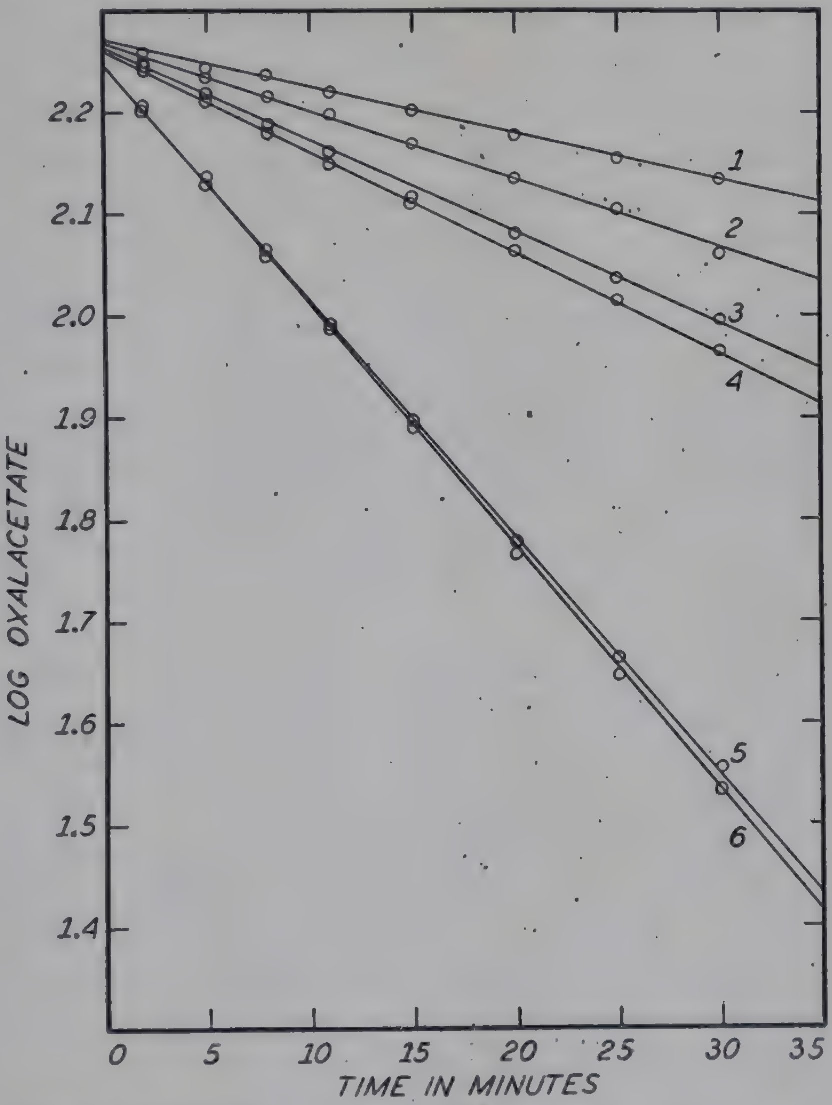

FIG. 3. Effect of malate and TPN on parsley root OAA carboxylase. 0.3 ml. of enzyme (17.4 mg. of net dry weight per ml.), 0.001 M $MnCl_2$; 0.025 M acetate, pH 5. Additions when indicated, 10 γ of TPN per 2 ml., 0.0125 M final concentration of malate.

Curve No.	k, min.$^{-1}$	Net k, min.$^{-1}$
1. Heated enzyme + malate	0.010	0
2. " "	0.015	0
3. Enzyme + malate	0.021	0.011
4. " + TPN + malate	0.023	0.013
5. " + "	0.055	0.040
6. "	0.054	0.039

Some typical results are shown in Fig. 3. In these experiments 0.001 M Mn^{++} was employed instead of 0.01 M Mn^{++}. Similar results are obtained, however, with higher Mn^{++} concentrations. The reaction rate constants for the various curves are shown in the figure legend. It is apparent tha TPN has no measurable effect either in the presence or in the absence o malate and that 0.0125 M malate causes a 70 per cent inhibition of the ne enzyme rate constant, but only 30 per cent inhibition of the heated blank

Because of recent evidence (15–18) that biotin may function as a cofacto for β-carboxylations, its possible relation to the plant enzyme was examined Added biotin has no influence on the action of the enzyme. Enzyme inactivated by exposure to pH 4.0 cannot be reactivated by biotin, nor does the presence of biotin influence the inactivation. The preparations have been found on analysis to contain biotin in somewhat variable amounts, bu there is no evidence that it is necessarily associated with the enzyme.

Below pH 6.0 and above pH 8.0, detectable inactivation of the carboxylase occurs within several hours at room temperature, and becomes more rapid as the pH is lowered or raised beyond these limits. Since the assay is run at pH 5.0, care must be taken not to delay the determination once the acetate buffer has been added to the enzyme. Lower activity values are obtained if the equilibration period is extended to half an hour instead of the usual 10 minutes.

When the enzyme solution is brought to pH 4.0 at 25°, 40 per cent inactivation occurs in 5 minutes, and below this pH complete inactivation is virtually immediate. This is associated with the appearance of a precipitate which redissolves as the pH is lowered further. On neutralization of such a solution, the precipitate reappears, and no enzyme activity can be detected. On the alkaline side 60 per cent inactivation was observed after a 5 minute exposure at 25° to pH 11.2, and 100 per cent inactivation at pH 11.6. The solution remains clear in the entire alkaline range and no precipitate forms on neutralization. At pH 7.4, samples held at 50°, 60°, and 70° for 5 minutes showed an inactivation of 25, 85, and 100 per cent respectively.

Reversibility of Oxalacetic Carboxylase—Evidence for the reversibility of oxalacetic carboxylase has previously been reported (10). OAA subjected to partial decarboxylation in the presence of enzyme, Mn^{++}, and $NaHC^{14}O_3$ can be shown to incorporate C^{14} into the β-carboxyl carbon. These experiments were conducted in a manner similar to those already described with pigeon liver enzyme (5), except that the reaction was stopped by addition of 2 ml. of 50 per cent citric acid. The incubation period was 8 minutes, in which time approximately half the added OAA had been decomposed.

The plant enzyme showed considerable exchange without addition of cofactors. Unlike the pigeon liver enzyme, ATP caused no increase in the exchange reaction, nor did TPN and adenylic acid. The results of these experiments are shown in Table I. In all cases, complete removal of $C^{14}O_2$ from the medium was demonstrated by negative "rinse" values, determined as previously described. The enzymic nature of the exchange reaction is shown by the virtual lack of exchange obtained with heated enzyme. In this experiment the incubation time was increased to 30 minutes to allow a comparable percentage of decarboxylation of OAA to be completed. The quantitative variation in results observed in the three experiments with active enzyme is probably not outside of the total limit of experimental error.

These experiments, together with the demonstration of the enzymic decarboxylation of OAA, demonstrate that reaction (*a*) with parsley root enzyme occurs in both directions, and that, in contrast to results with the pigeon liver enzyme, neither ATP nor TPN appears to affect this reaction appreciably.

TABLE I

Fixation of $C^{14}O_2$ in Oxalacetic Acid during Enzymic Decarboxylation by Parsley Root Preparation

Initial reaction mixture, 4.25×10^{-2} M oxalacetate, 0.001 M $MnCl_2$, 1.2×10^{-2} M phosphate buffer, pH 6.0, 3.6×10^{-3} M $NaHCO_3$ containing C^{14}; 180 mg. of lyophilized parsley root protein and the cofactors indicated in the table in a total volume of 10 ml. Incubated for 8 minutes at 30°.

Experiment No.	Special conditions	Added cofactors	Residual oxalacetate*	Radioactivity† of β-carboxyl carbon, as per cent of activity of bicarbonate + carbonic acid of medium
			μl. CO_2	
1	None		5120‡	0.49 ± 0.02
2	"	3.0×10^{-4} M ATP	5400‡	0.52 ± 0.03
3	Enzyme solution heated; reaction time, 30 min.	3.0×10^{-4} " "	7600	0.04 ± 0.02
4	None	5.0×10^{-4} M TPN 2.9×10^{-4} " adenylic acid	6600‡	0.31 ± 0.03

* Initial oxalacetate, approximately 9300 μl.

† The error given is the probable error (statistical).

‡ Approximately 15 per cent of oxalacetate decarboxylation is non-enzymatic and due to Mn^{++} alone.

Since the enzyme preparation contains a malic dehydrogenase active with TPN, it is also possible to demonstrate the combined action of the carboxylase and of the dehydrogenase (reaction (*a*) + (*b*)) by using the spectrophotometric procedure of Ochoa. The reduction of TPN by added malate is followed by measuring the increase in light absorption at 340 mμ due to formation of $TPN_{red.}$. In the presence of added Mn^{++} the reaction proceeds to the right according to the equation

(*e*) $\text{Malate} + \text{TPN}_{\text{ox.}} \rightarrow \text{TPN}_{\text{red.}} + \text{pyruvate} + \text{CO}_2$

The addition of pyruvate and CO_2 after the reduction has ceased results in a reoxidation of $\text{TPN}_{\text{red.}}$, indicating that the reaction is freely reversible.

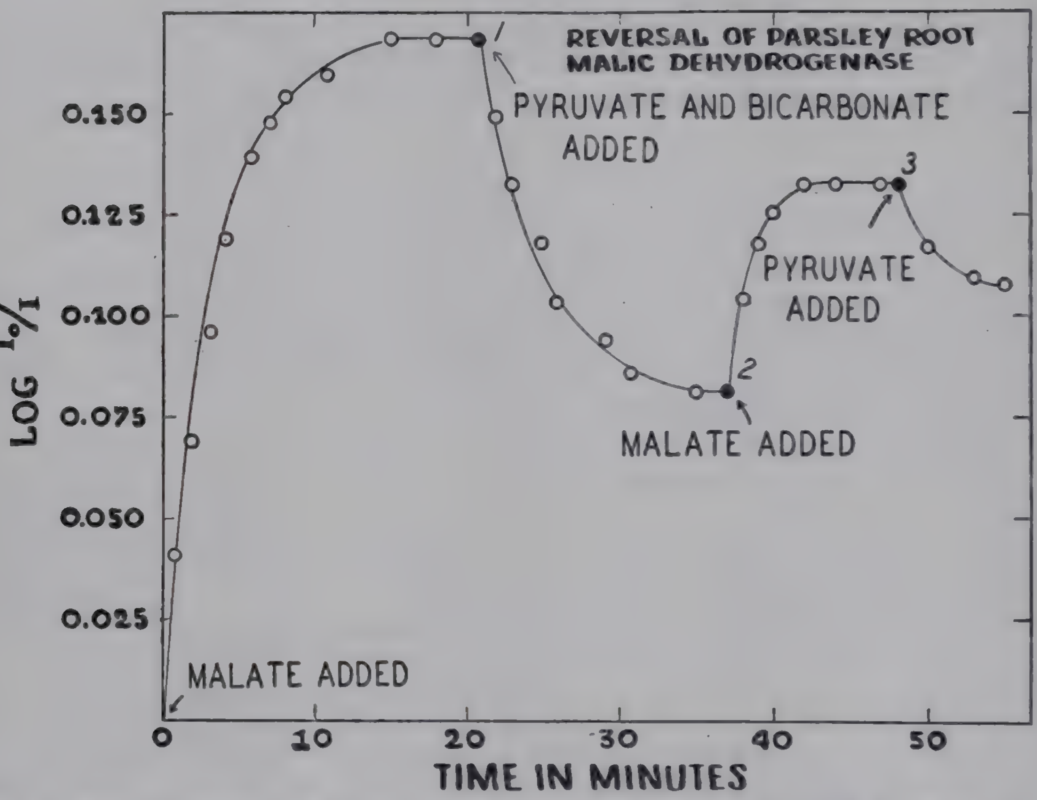

FIG. 4. The reversal of malic dehydrogenase by pyruvic acid and CO_2 in the presence of a parsley root preparation. Composition of solution, 0.03 M glycylglycine buffer, pH 7.4, 0.0012 M $MnCl_2$, 1.1 mg. of parsley root preparation, and 408 γ of TPN in a total volume of 3.2 ml. At time 0 add 0.001 mM of *l*-malate; at *1* add 0.05 mM of pyruvate plus 0.3 ml. of 0.1 M $NaHCO_3$ saturated with CO_2; at *2* add 0.0025 mM of *l*-malate; at *3* add 0.05 mM of pyruvate. Absorption corrected for change in volume. Readings made against a blank which contained all components except TPN.

Reduction and oxidation may be repeated at will by addition of appropriate amounts of substrate. A typical experiment of this type is shown in Fig. 4.

Ochoa *et al.* (9) have shown that oxalacetate cannot reoxidize $\text{TPN}_{\text{red.}}$ in experiments of this sort conducted with purified pigeon liver enzyme. Reaction (*e*) is accordingly regarded as not proceeding by way of OAA. In the case of the plant enzyme, OAA is capable of reoxidizing $\text{TPN}_{\text{red.}}$ with the preparations tested. It seems appropriate, therefore, to consider reaction (*e*) as the sum of reactions (*a*) and (*b*). However, since the plant preparation with which this experiment has been conducted is relatively crude, the possibility must not be overlooked that some of the apparent properties of the enzymes may change on purification.

Supplementary evidence for the occurrence of reaction (*e*) in parsley root is afforded by the incorporation of C^{14} into the organic acid fraction when the parsley root enzyme is incubated with unlabeled pyruvate and malate in the presence of $C^{14}O_2$. The results of such an experiment, presented in Table II, show that the C^{14} fixed in the organic acid fraction is proportional to the malate content of the fraction. The conditions of the experiment are indicated in Table II. After the reaction mixture had been incubated for 3 hours, the experiment was terminated by addition of metaphosphoric acid. $C^{14}O_2$ was removed, the protein precipitate was centrifuged, and the acidified supernatant was subjected to continuous extraction with three successive portions of ether. The dry materials in the extracts were analyzed for *l*-malate[3] and pyruvate (19), and measured for radioactivity.

TABLE II

Fixation of $C^{14}O_2$ in Organic Acids during Incubation of Parsley Root Protein with l-Malic and Pyruvic Acid Substrates

Initial reaction mixture, 0.05 M phosphate buffer, pH 6.7, 0.017 M *l*-malate, 0.034 M pyruvate, 0.01 M $MnCl_2$, 7.5×10^{-4} M ATP, 2.4×10^{-3} M $NaHCO_3$ containing C^{14} (thick sample count, 9000 per minute); 235 mg. of lyophilized parsley root protein and 19 γ of TPN in a total volume of 15 ml. Deproteinization with metaphosphoric acid yielding 17 ml. of supernatant and 3 ml. of precipitate.

		Composition of extracted materials			Radioactivity*
Ether extract (1)	Time of extraction (2)	Total dry weight (3)	Sodium malate (4)	Sodium pyruvate (5)	Count per mM sodium malate in thick sample, corrected for dilution effect†
	hrs.	*mg.*	*mg.*	*mg.*	*counts per min. per mM*
1st	12 (Slowly)	160	13.3	13.5	650 ± 30
2nd	2 (Rapidly)	110	13.3	0.74	680 ± 20
3rd	12 (Slowly) 4 (Rapidly)	14.7	4.27	0	570 ± 30

* The error given is the statistical probable error.

† Calculated from thick sample counts and malate concentration. Thick sample counts first corrected for dilution factor given by (Column 3)/(Column 4). Thick sample, 20 mg. per sq. cm., 3.47 sq. cm. total area. Therefore thick sample = 69.4 mg. or 0.387 mM of sodium malate.

That the amounts of total C^{14} fixed in such an experiment are relatively small has since been found to be due, apparently, to the destruction of TPN. Though incubation was continued for 3 hours, most of the fixation probably occurred within the first half hour. It is necessary to add considerably larger amounts of TPN to achieve higher fixation rates. The action of the ATP seems to be to cause a partial prevention of the TPN destruction.[2]

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[3] Speck, J. F., unpublished method.

Carboxylase Activity of Crystalline Globulins from Cucurbit Seeds—It has previously been reported (2) that the crystalline globulins which occur in high concentration in squash and pumpkin seeds (20) possess oxalacetic carboxylase activity. This activity is largely heat-labile, though heat-denatured protein retains a definite catalytic effect on the decarboxylation. Divalent cations have no effect on the enzyme action, since their presence accelerates decarboxylation with both unheated and heat-inactivated protein to the same extent. Under the conditions of the standard test system, but with Mn^{++} omitted, four times recrystallized globulin shows an activity per mg. of protein of the same order of magnitude as the crude unpurified preparations from parsley root. The globulin is virtually inactive at pH 5.5, however, at which it is practically insoluble. At pH 5.0 it still remains largely as a precipitate in the test system, but at pH 4.5 it goes into solution and the activity is considerably increased. It appears to us that these globulins, which must be regarded largely as storage proteins, possibly do not possess any physiological function in so far as the carboxylation reaction is concerned. It may be noted that they are inactive at physiological pH values and that their activity is relatively low compared to the cation-activated carboxylases. Furthermore, it has been possible to demonstrate that there is present in *Cucurbita* seeds, in addition to the globulin, a separate water-soluble oxalacetic carboxylase which is cation-activated and appears to be similar in nature to the β-carboxylase of parsley root (12).

DISCUSSION

It seems probable that the reversible carboxylation of oxalacetate plays an important rôle in the conversion of carbohydrate to dicarboxylic acids, and our results indicate that the process in plants is similar in outline, if not in detail, to that which occurs in liver tissue. Parsley root, like pigeon liver, contains a malic dehydrogenase active with TPN, together with an OAA carboxylase. It is possible that the two activities may be associated with 1 molecule, as Ochoa has suggested for the liver enzymes. The association of a TPN-active malic dehydrogenase with OAA carboxylase seems to occur very frequently in plant preparations. Final judgment on the significance of this association must await purification of the various activities.

However, in spite of the basic similarities of the parsley root and pigeon liver enzymes, there are certain decided differences among the characteristics of the two preparations. These may be summarized as follows:

First, the kinetics of the decarboxylation of OAA by the pigeon liver enzyme and the plant enzyme are apparently different. With relatively small amounts of OAA, the pigeon liver enzyme shows zero order kinetics; the plant enzyme, first order kinetics, with respect to substrate. The optimum Mn^{++} concentration is likewise different, being 0.01 M for the parsley root enzyme and 0.001 M for the pigeon liver enzyme. Both these differences imply that the pigeon liver enzyme combines more readily with both substrate and Mn^{++} than does the plant enzyme. This interpretation is further substantiated by the fact that the plant enzyme can readily be freed by dialysis (12 hours) from all cation activators present in the initial extracts, whereas the pigeon liver enzyme present in acetone powder extracts retains considerable heat-labile activity without added divalent cations, even after prolonged dialysis (4 days).

The differences in the effect of ATP and of TPN on the two enzymes are perhaps even more striking. The OAA carboxylase of pigeon liver shows a decided stimulation of decarboxylation by TPN, whereas the exchange of CO_2 with the β-carboxyl of OAA requires the addition of ATP. These apparently anomalous effects have not been satisfactorily explained, though they would appear to be related to the association of the malic dehydrogenase with the carboxylase. In the case of the plant enzyme, TPN and ATP have no effect on the decarboxylation reaction or the exchange reaction. The possibility that these characteristics may change on purification must of course not be overlooked. This has already been mentioned in connection with the fact that the reoxidation of reduced TPN is readily effected by OAA in the presence of the plant enzyme, whereas such a reaction is not catalyzed by the purified pigeon liver enzyme of Ochoa.

With the reservation in mind that data obtained on impure preparations are not final, it nevertheless appears appropriate to emphasize that the plant enzyme can apparently catalyze both reactions (*a*) and (*b*) independently

$$(a) \qquad \text{Oxalacetate} \rightleftarrows \text{pyruvate} + CO_2$$
$$(b) \qquad l\text{-Malate} + TPN_{ox.} \rightleftarrows \text{oxalacetate} + TPN_{red.}$$

and it is therefore unnecessary to regard reaction (*c*)

$$(c) \qquad \text{Malate} + TPN_{ox.} \rightleftarrows \text{pyruvate} + CO_2 + TPN_{red.}$$

as anything but the sum of reactions (*a*) and (*b*). The pigeon liver enzyme on the other hand, as pointed out by Ochoa, seems to catalyze reactions (*a*) and (*c*), but not reaction (*b*). Ochoa has suggested that the intermediary OAA is metabolized on the surface of the enzyme where it is produced. Perhaps the differences between pigeon liver and parsley root enzymes may be related to their different degrees of combination with the substrate OAA. The Michaelis constants have not been determined for either enzyme. However, a zero order reaction implies a Michaelis constant too small to be measurable, and a first order reaction, a Michaelis constant too large to be measurable (at least in the range in which the reaction remains first order). Consequently, the pigeon liver enzyme would be expected to combine much more firmly with OAA than would the plant enzyme.

It does not seem probable that all the differences noted between plant and animal enzymes can be attributed to the effects of adventitious impurities, but it appears more likely that pigeon liver enzyme and parsley root enzyme are different proteins with similar function. Whether these β-keto carboxylases may be regarded as typical of animal and plant carboxylases respectively remains to be determined.

SUMMARY

1. The preparation of an oxalacetic carboxylase from parsley root and its quantitative assay are described.

2. The enzyme has been shown to be associated with a malic dehydrogenase active with TPN.

3. The reversibility of the oxalacetic carboxylase has been demonstrated.

4. Some properties of the oxalacetic carboxylase activity of crystalline globulins from squash and pumpkin seeds are discussed.

5. The properties of parsley root oxalacetic carboxylase have been compared with those of pigeon liver oxalacetic carboxylase.

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THE EFFECT OF CATIONS ON THE DECARBOXYLATION OF OXALACETIC ACID*

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In connection with studies on the β-keto acid carboxylases of plants (1, 2), the effects of metal ions on the decarboxylation of oxalacetic acid, in the presence and absence of enzyme, were studied. In confirmation of Krebs (3) the decarboxylation was found to be accelerated by a variety of polyvalent cations. It was also observed that the enzyme oxalacetic carboxylase from parsley roots, which is nearly inactive in the absence of metal ions, is activated by a variety of divalent cations.

Methods

The experimental procedures used in studying keto acid decarboxylation are described in the preceding paper (2). Evolution of carbon dioxide from oxalacetic acid was measured in Warburg manometers, with a reaction mixture buffered at pH 5 to avoid retention. Since in most cases the decarboxylation reactions follow first order kinetics with respect to oxalacetic acid, the rates are conveniently expressed as first order reaction rate constants.

Results

Effect of Cations on Non-Enzymatic Decarboxylation of Oxalacetate—All the polyvalent cations tested, with the exception of Ba^{++}, accelerate the decarboxylation of oxalacetate. Monovalent cations were not studied, but Krebs (3) found them to be ineffective. In agreement with Krebs, it was noted that the effect of the cation is independent of the nature of the anion added with it, provided the salt is ionized. Except in the case of Fe^{+++} and Al^{+++}, which are described later, the decarboxylation reactions follow first order kinetics with respect to oxalacetic acid. When Cu^{++} is present in concentrations of 0.01 M or greater, the first order constants tend to decrease somewhat with time, and the average has been taken.

In Fig. 1 the first order rate constants are plotted against the logarithms

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of the molarities of the cations. The curves for the various ions are similar in shape, but the concentration giving a maximum effect and the magnitude of the effect vary with different cations. Cu^{++} and La^{+++} give maxima at concentrations of 0.001 M. The activity maximum shown for Pb^{++} may not be similar in nature, because in samples containing Pb^{++} at concentrations of 0.01 M or greater a precipitate (perhaps lead oxalacetate) appears on tipping in the oxalacetic acid. Other ions, such as Zn^{++}, Ni^{++}, Co^{++}, and Fe^{++}, tend to approach maxima at the highest concentrations studied.

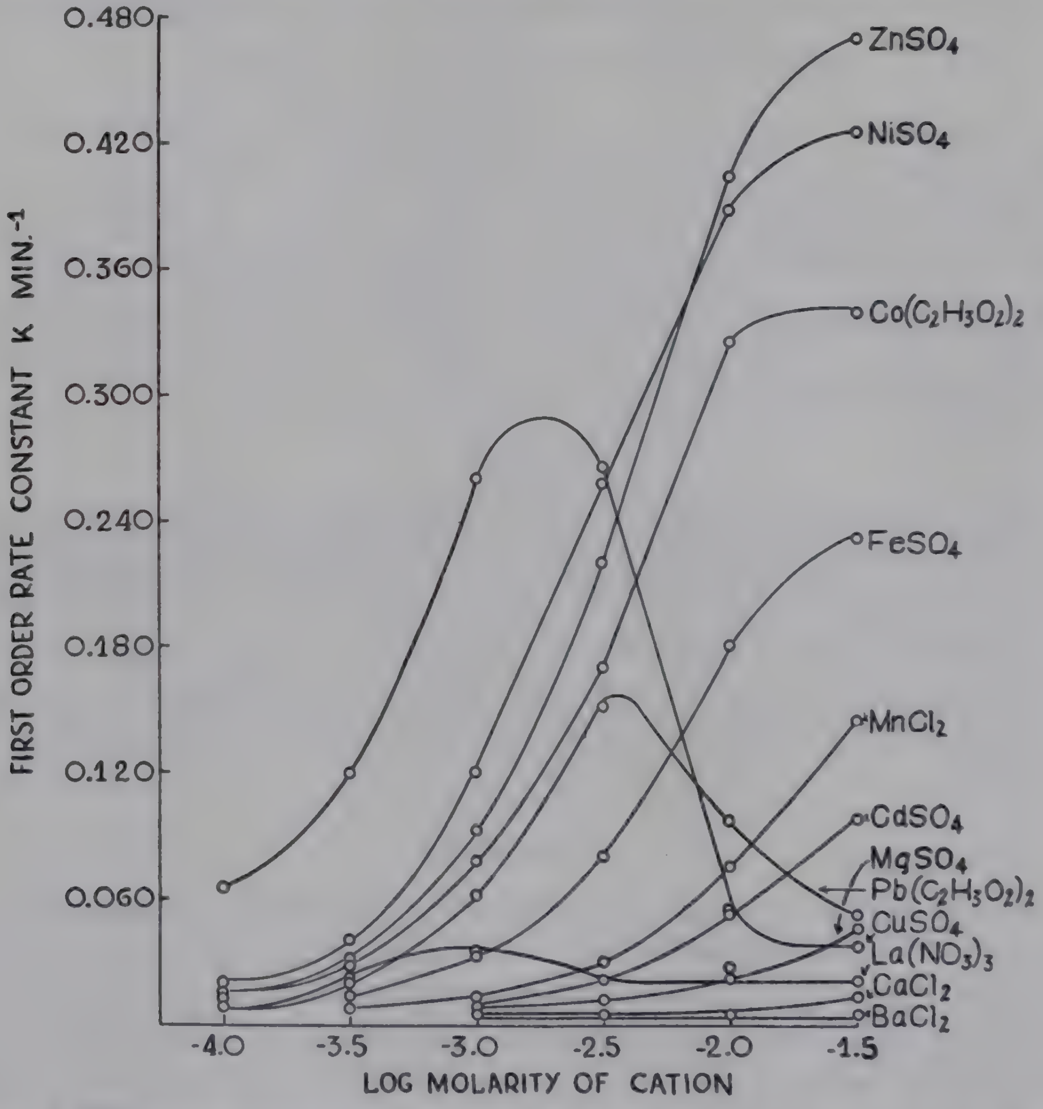

Fig. 1. Effect of various cations on the non-enzymatic decarboxylation of oxalacetate. Samples contained 0.1 M acetate, pH 5.0, 1 mg. of oxalacetic acid (equivalent to 160 μl. of CO_2), and metal salts in the concentrations indicated, in a total volume of 2.0 ml. Temperature 30°. In the absence of added polyvalent cation, the first order rate constant was 0.006 $min.^{-1}$.

It was necessary to employ anaerobic conditions in testing the action of Fe^{++} ions. When air was used as the gas phase, a yellow color (probably due to formation of basic ferric acetate) appeared at the moment when the oxalacetic acid was tipped in. Subsequently oxygen was taken up at the same time that carbon dioxide was liberated, and the kinetics of the reaction were complex. Under anaerobic conditions (nitrogen as the gas phase, yellow phosphorus in the center well), no yellow color appeared on tipping in oxalacetic acid, and the decarboxylation followed first order kinetics.

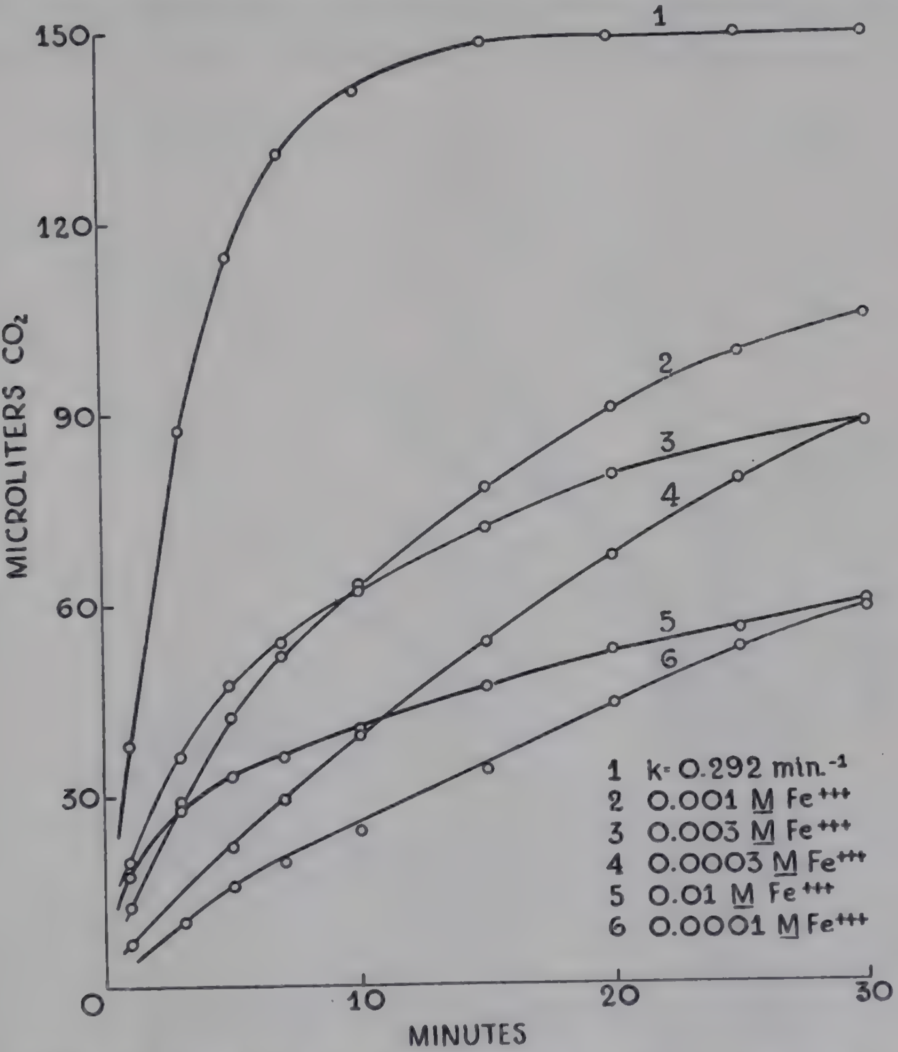

FIG. 2. Effect of Fe^{+++} on the non-enzymatic decarboxylation of oxalacetate. Samples contained 0.1 M acetate, pH 5.0, oxalacetic acid equivalent to 150 μl. of CO_2, and $FeNH_4(SO_4)_2$ in the concentrations indicated, in a volume of 2.0 ml. Temperature 30°; gas phase N_2. Curve 1 was calculated for a first order rate constant of 0.292 $min.^{-1}$, corresponding to the maximum rate with Cu^{++}.

Fe^{+++} and Al^{+++} gave results different from those obtained with the other ions, and these effects are represented in Figs. 2 and 3. Decarboxylation of oxalacetate in the presence of these ions follows first order kinetics only at low cation concentrations (below 0.001 M). At higher concentrations the rates fall off more rapidly than expected for first order reactions, particularly in the case of Fe^{+++}. Both ions give maximum rates of decarboxylation at a concentration of about 0.001 M, and the maximum rates are lower than those observed with the most effective divalent cations, such as Zn^{++} and Ni^{++}. The experiments with Fe^{+++} were performed under anaerobic conditions.

Effect of Cations on Enzymatic Decarboxylation of Oxalacetate—A preparation of oxalacetic carboxylase from parsley roots, made as described in the preceding paper, was used in studying the effect of cations on the enzymatic decarboxylation of oxalacetate. In the absence of added divalent cations, decarboxylation of oxalacetate by the enzyme is very slow. All the divalent cations studied activate the enzyme; *i.e.*, the rate of decarboxylation of oxalacetate in the presence of enzyme plus cation is greater than in the presence of cation alone, of enzyme alone, or of cation plus heat-inactivated enzyme. In all cases of activation, the reactions follow first order kinetics.

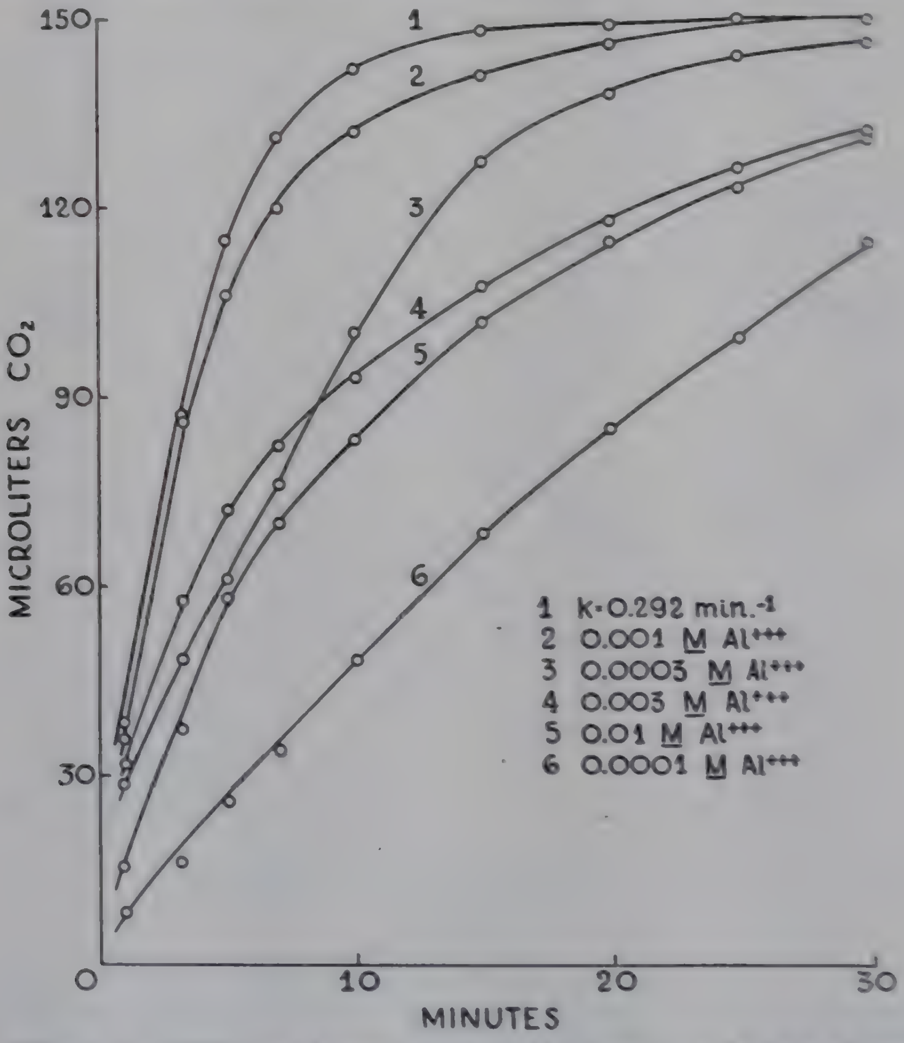

FIG. 3. Effect of Al^{+++} on the non-enzymatic decarboxylation of oxalacetate. Samples contained 0.1 M acetate, pH 5.0, oxalacetic acid equivalent to 150 μl. of CO_2, and $Al_2(SO_4)_3$ in the concentrations indicated, in a volume of 2.0 ml. Temperature 30°. Curve 1 was calculated for a first order rate constant of 0.292 $min.^{-1}$.

La^{+++} ions do not activate the enzyme, and Fe^{+++} and Al^{+++} ions give complex results, which are described later.

In Fig. 4 the relative activity of the parsley root enzyme in the presence of different cations is plotted against the logarithm of the cation concentration. The various ions give curves of similar shape which differ from each other in the position and height of the maximum. All of the ions except Mg^{++} and Ba^{++} show maximum effects within the range of concentrations studied. The maximum for Ca^{++} actually lies just at the highest concentration, 0.03 M; higher levels give lower enzyme activities. Of the ions tested, Mn^{++} is most effective in activating the parsley root enzyme.

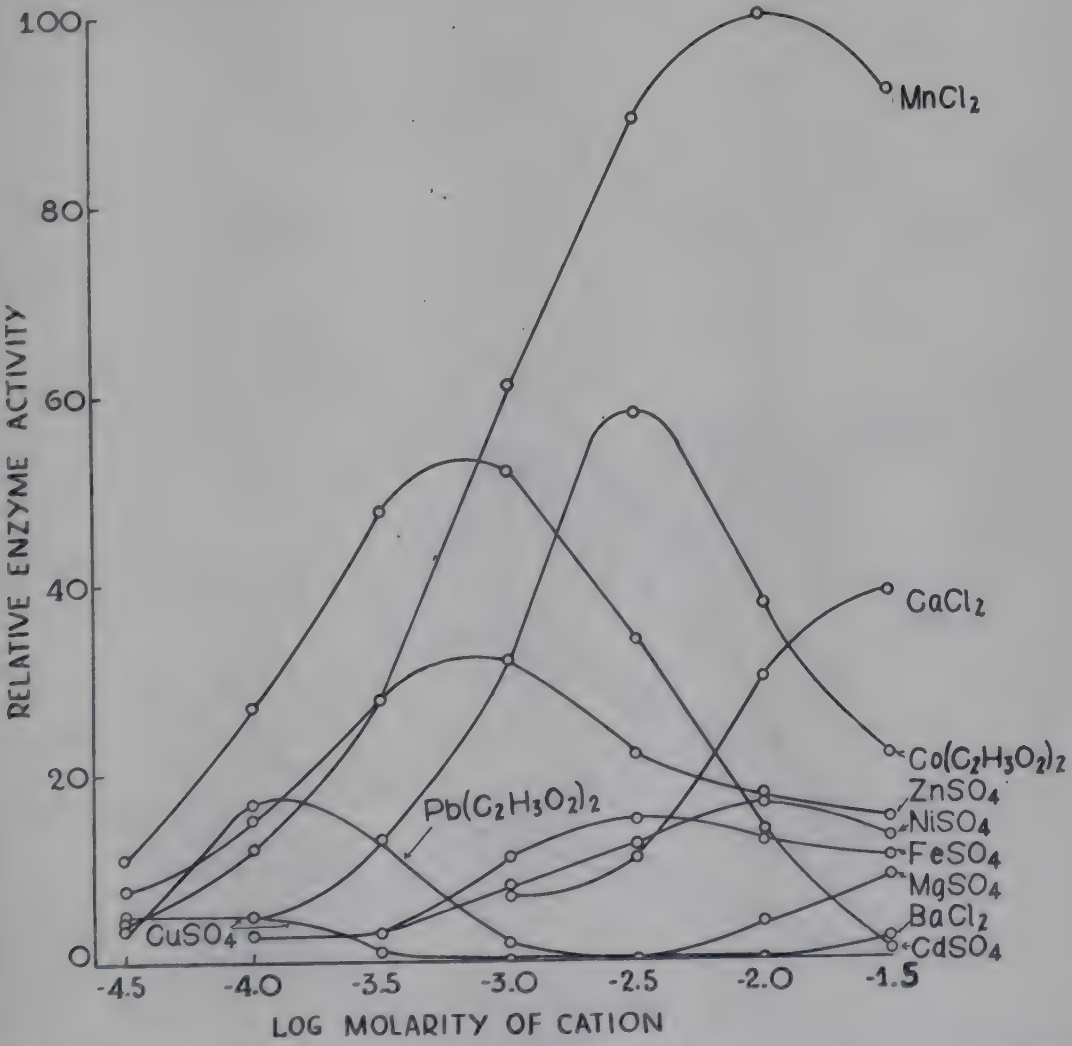

FIG. 4. Effect of various cations on the enzymatic decarboxylation of oxalacetate Samples contained 0.1 M acetate, pH 5.0, 1 mg. of oxalacetic acid, 15 mg. of lyophilized parsley root enzyme, and various ions in the concentrations indicated, in a volume of 2.0 ml. Temperature 30°. The rate with 0.01 M Mn^{++} is taken as 100. The relative rate in the absence of added divalent cations was 1.

In most cases the first order rate constants for the decarboxylation of oxalacetate in the presence of cation plus heat-inactivated enzyme are the same as in the presence of an equal concentration of cation alone. This indicates that the inactivated protein does not firmly bind these ions. However, the heat-inactivated protein reduces the rates obtained with Co^{++} and Ni^{++} at 0.03 M concentrations and with Zn^{++} at concentrations of 0.003 M and above. Decarboxylation of oxalacetate in the presence of heated enzyme and La^{+++} or Pb^{++} occurs at the same rate as that observed in the absence of added metal ions; the inactive protein abolishes the catalytic activity of these cations, although Pb^{++} activates the unheated enzyme.

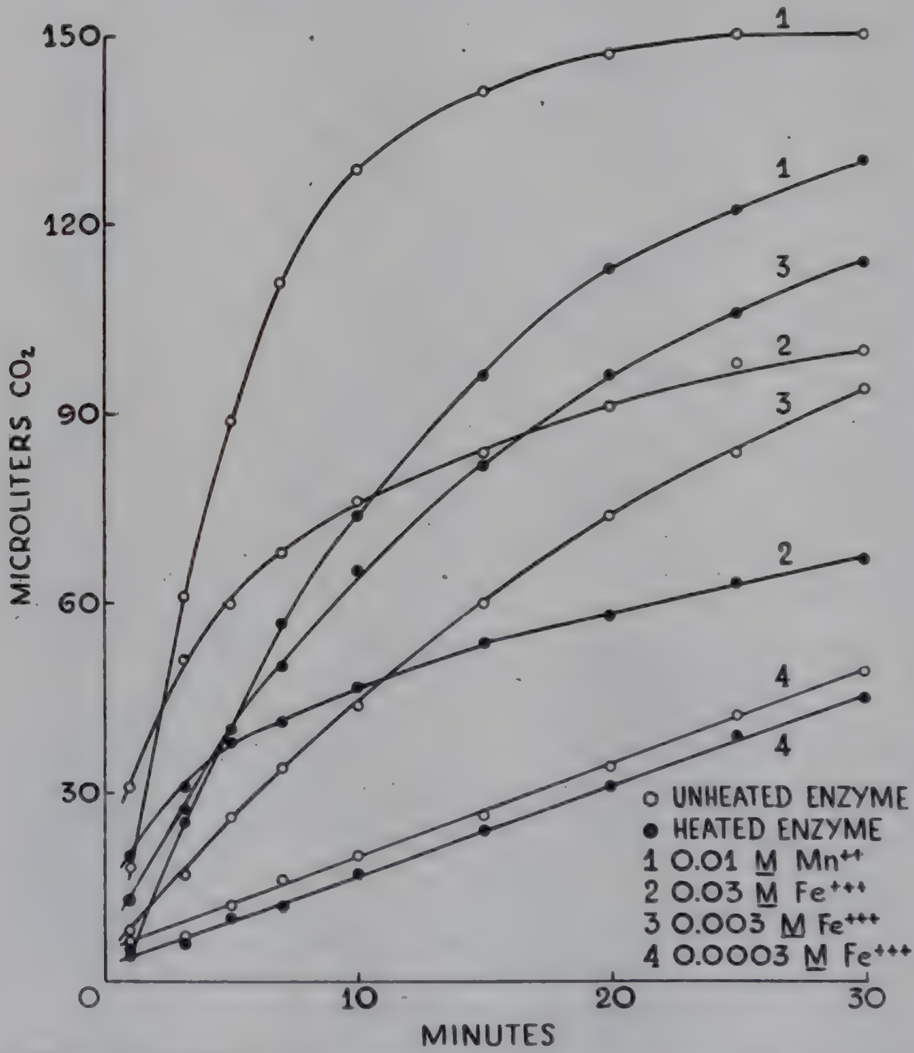

FIG. 5. Effect of Fe^{+++} on the enzymatic decarboxylation of oxalacetate. Samples contained 0.1 M acetate, pH 5.0, oxalacetic acid equivalent to 150 µl. of CO_2, 15 mg. of lyophilized parsley root enzyme, and $MnCl_2$ or $FeNH_4(SO_4)_2$ in the concentrations indicated, in a total volume of 2.0 ml. Temperature 30°.

Fe^{+++} and Al^{+++} gave anomalous results, which are represented in Figs. 5 and 6. Decarboxylation of oxalacetate in the presence of these ions does not follow first order kinetics. At cation concentrations of 0.0001 and 0.0003 M the rates in the unheated samples are the same as or slightly greater than in the heated samples; both rates are considerably lower than those observed with the ions in the absence of any protein (compare with Figs. 2 and 3). At cation concentrations of 0.001 and 0.003 M the rates in the heated samples are equal to or greater than those in the unheated samples; both rates are somewhat lower than those observed in the absence of protein. At cation concentrations of 0.01 and 0.03 M, the rates in the unheated samples are the same as or greater than in the heated samples; both rates are greater than those found in the absence of protein. An explanation for these results is not apparent. When Fe^{+++} was present in high concentrations, some tendency to form deposits of basic ferric acetate on protein particles was noted. In any case, it seems unlikely that either Fe^{+++} or Al^{+++} activates the parsley root carboxylase significantly.

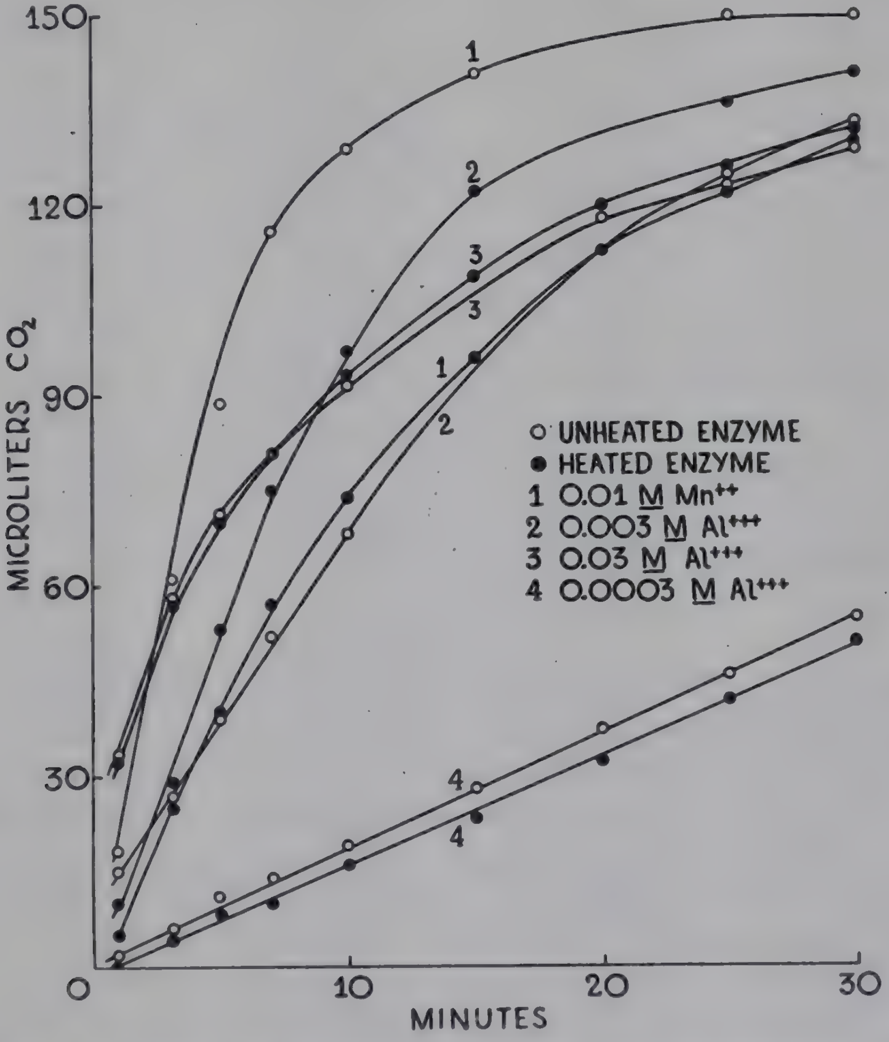

FIG. 6. Effect of Al^{+++} on the enzymatic decarboxylation of oxalacetate. Samples contained 0.1 M acetate, pH 5.0, oxalacetic acid equivalent to 150 μl. of CO_2, 15 mg. of lyophilized parsley root enzyme, and $MnCl_2$ or $Al_2(SO_4)_3$ in the concentrations indicated, in a total volume of 2.0 ml. Temperature 30°.

The activity of the carboxylase with mixtures of two different cations, each in suboptimum concentration, was studied. In the presence of heated enzyme the effects produced by Zn^{++} and Cd^{++} are partially additive. The following first order rate constants were observed: 0.012 $min.^{-1}$ with 0.001 M $CdSO_4$, 0.030 $min.^{-1}$ with 0.0003 M $ZnSO_4$, and 0.037 $min.^{-1}$ with both. However, these cations compete in activating the unheated enzyme, so that intermediate activity is observed with both present. The relative enzyme activities (activity with 0.01 M $MnCl_2$ taken as 100) were 39 with 0.001 M $CdSO_4$, 23 with 0.0003 M $ZnSO_4$, and 35 with both.

Experiments were carried out in which the acetate buffer was replaced by benzoate, phthalate, oxalate, succinate, tartrate, or citrate of the same pH and molar concentration. 0.001 M $MnCl_2$ was present, and the rate of decarboxylation of oxalacetate was measured with and without enzyme. All of the buffers except benzoate reduce the rate of non-enzymatic decarboxylation below the level observed with 0.001 M Mn^{++} in acetate, and added enzyme is completely inactive. In the case of benzoate, the non-enzymatic rate is the same as with 0.001 M Mn^{++} in acetate, but added enzyme is inactive. Probably phthalate, oxalate, succinate, tartrate, and citrate decrease the decarboxylation rate by forming complexes with Mn^{++}, while benzoate more specifically inhibits the enzyme.

DISCUSSION

Krampitz and Werkman (4) first described the acceleration of the non-enzymatic decarboxylation of oxalacetate by metal ions for the particular case of Mg^{++}, and Krebs (3) investigated this effect of cations in greater detail. The results of his studies and of the experiments reported in the present paper may be summarized as follows: Many polyvalent cations accelerate the decarboxylation of oxalacetate. Of those tested, Zn^{++} and Ni^{++} are most effective. Anions and monovalent cations do not influence the reaction. This action of cations is general for the decarboxylation of β-keto dicarboxylic acids, such as oxalacetate, acetonedicarboxylate, and oxalosuccinate (5). Non-enzymatic decarboxylation of α- or β-keto monocarboxylic acids is not affected by metal ions.

Enzymes which catalyze the decarboxylation of oxalacetate have been found in bacteria (4, 6), animal tissues (7), and plants (1). All these enzymes require metal ions for full activity, and Mn^{++} has nearly always been employed. However, complete studies on the cation specificity of oxalacetic carboxylases from various sources have not been made. The carboxylase from parsley roots is activated by a considerable number of divalent positive ions, including Cu^{++}, Pb^{++}, Ba^{++}, Mg^{++}, Fe^{++}, Ni^{++}, Zn^{++}, Ca^{++}, Cd^{++}, Co^{++}, and Mn^{++}; Mn^{++} is most effective. There is no obvious correlation between the activities of the ions in the presence and absence of enzyme, but it appears that maximum activation of the enzyme is achieved at lower concentrations than are required for maximum rates of decarboxylation in the absence of enzyme. A similar carboxylase prepared from the red radish is active in the presence of Pb^{++}, Ni^{++}, Zn^{++}, Mg^{++}, Cd^{++}, Co^{++}, and Mn^{++}; Mn^{++} again gives the most rapid rates.[1] Oxalacetic acid carboxylase from pigeon liver is activated by Mn^{++} (7) and less effectively by Co^{++}.[2] The enzyme from *Micrococcus lysodeikticus* functions with Mg^{++} or Mn^{++} (4, 8) and that from *Escherichia coli* with Mn^{++} (6).

The concentrations of divalent cations in intact plant tissues are probably not sufficiently high to permit maximum activity of oxalacetic carboxylase unless the ions are localized at the site of enzyme action. For example, the Mn^{++} content of parsley and parsnip roots is only about 0.075 mM per 1000 gm. of fresh weight.[3] A number of the cations which activate the plant carboxylases are essential nutrients for plants; for example, Cu^{++}, Mg^{++}, Fe^{++}, Zn^{++}, Ca^{++}, and Mn^{++} (10). These ions may function in part as cofactors for oxalacetic carboxylase or other enzymes of wide distribution and possible great importance in plant metabolism.

Kornberg, Ochoa, and Mehler (8) have recently presented evidence that the effect of cations such as Al^{+++} and Mn^{++} on oxalacetate decarboxylation is due to formation of an unstable cation-oxalacetate complex, which decomposes to give pyruvate, carbon dioxide, and free cation. The carboxylase protein appears to accelerate the formation or breakdown of the complex. A number of cations seem capable of forming such complexes with oxalacetate; the affinity between metal ions and oxalacetate and the rate of decomposition of the complex vary with the different metal ions. The ability of enzymes to accelerate the formation or breakdown of certain of these complexes introduces a new element of specificity into the effects, since enzymes from different sources may vary in their activity with different ions.

SUMMARY

Decarboxylation of oxalacetic acid in the presence of polyvalent cations follows first order kinetics. The rates of decarboxylation with fourteen different ions over a range of concentrations are reported. The enzyme oxalacetic carboxylase from parsley roots is activated by a number of divalent cations, of which Mn^{++} is most effective. The relative enzyme activities with different concentrations of these metal ions are given.

[1] These experiments were performed by Miss Miriam C. Gollub of this department.

[2] Personal communication from Dr. Birgit Vennesland.

[3] The analyses were kindly carried out by Dr. Ernest Kun, using a procedure which he has recently described (9).

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A GENERAL METHOD FOR THE SYNTHESIS OF α,γ-DIAMINO ACIDS*

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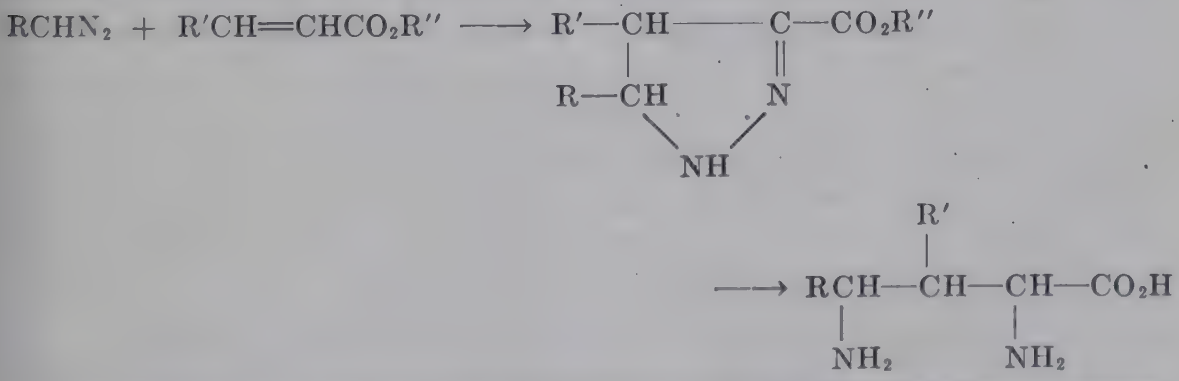
Carter and his associates (1, 2) have described the degradation of streptamine to α,γ-diamino-β-hydroxyglutaric acid. In order to compare this product with a compound obtained synthetically, a study of the preparation of α,γ-diamino acids was undertaken. Of particular interest was α,γ-diaminoglutaric acid, which might be expected to result from further degradation of α,γ-diamino-β-hydroxyglutaric acid. Another substance prepared in the course of this work, α,γ-diaminobutyric acid, is of current interest as a result of the discovery of its presence in the antibiotic substances polymyxin and aerosporin.

A survey of the literature revealed that no general method was available for the synthesis of α,γ-diamino acids and that very few compounds of this type were known. A number of investigators (3–7) have described the preparation of α,γ-diaminobutryic acid (L and DL forms). However, the methods employed were tedious, gave poor yields, and were not generally applicable.

In considering possible methods of synthesis of α,γ-diamino acids, the reduction of pyrazoline-3-carboxylic esters appeared promising, especially since a wide variety of pyrazolines is readily available.



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This reaction has received but little attention. Balbiano (8), in 1888, reported the reduction of pyrazolines to diamines with sodium and alcohol, but the possible synthetic applications of this reaction have not been investigated. Thoms and Schnupp (9) found that the catalytic reduction of pyrazoles proceeded only to the pyrazolidine stage. However, it seemed likely that further reduction to the diamine might be effected under the proper conditions and an investigation of this reaction was undertaken.

It was discovered that the reduction of pyrazoline-3-carboxylic esters proceeds smoothly and rapidly at high pressures of hydrogen with Raney's nickel catalyst. From the reduction product excellent yields (usually over 80 per cent) of α,γ-diamino acids were obtained. The compounds prepared in this way include α,γ-diaminobutyric acid, α,γ-diamino-β-methylbutyric acid, α,γ-diamino-β-phenylbutyric acid, α,γ-diaminoheptanoic acid, and α,γ-diaminoglutaric acid.

In certain cases the first 2 atoms of hydrogen were absorbed readily, probably with the formation of a pyrazolidine,[1] and more strenuous conditions were required to complete the reduction. After removal of the catalyst the reaction mixtures were usually clear and colorless, indicating that little or no side reaction had occurred. The primary reduction products were hydrolyzed with aqueous hydrochloric acid and the α,γ-diamino acids were isolated as hydrochlorides, except in the case of α,γ-diaminoglutaric acid which was obtained as the free amino acid.

In every case except α,γ-diaminobutyric acid, two racemic forms are possible. Therefore, the reduction products were examined carefully for homogeneity. It was possible to separate two racemic forms of α,γ-diaminoheptanoic acid by fractionation of the crude monohydrochlorides from aqueous alcohol-ether mixtures. α,γ-Diamino-β-phenylbutyric acid monohydrochloride also appeared to consist of a mixture of isomers in approximately equal amounts. The major portion of the crude α,γ-diamino-β-methylbutyric acid consisted of a single racemic form. The second isomer (if present) was not isolated. α,γ-Diaminoglutaric acid was examined with special care for the possible presence of isomeric forms. In one reduction an 88 per cent yield of the crude diamino acid was obtained from dimethyl pyrazoline-3,5-dicarboxylate. 75 per cent of this material was obtained in a purified, apparently homogeneous state. This product gave a dibenzoyl derivative (78 per cent yield) melting at 206–209°, which on recrystallization from hot water showed no evidence of fractionation. The dibenzoyl derivative on treatment with diazomethane gave a dimethyl ester in almost quantitative yield. The ester melted sharply in a capillary tube (139–141°), but on the block under a polarizing microscope two types of crystals were observed. Careful fractionation of this material led to the separation of two isomers, one melting at 152–153°, the second at 179–180°. The former is more soluble in cold acetone than the latter but has approximately the same solubility in acetone-hexane mixtures. This differential solubility behavior provided a satisfactory basis for fractionation of mixtures of the two methyl esters. The esters melting at 152–153° and 179–180° yielded dibenzoylaminoglutaric acids melting at 203–204° and 215–217° respectively. There is very little depression of melting point on mixing the two acids.

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[1] Catalytic reduction of hydrazones and azo compounds usually proceeds readily to the hydrazine stage, but cleavage of the latter may require more drastic conditions.

The two isomeric ethyl α,γ-dibenzoylaminoglutarates (m.p. 130–132° and 158–160°) were separated in the same manner as the dimethyl esters.

In a later run a small quantity of dimethyl pyrazoline-3,5-dicarboxylate was reduced with a higher ratio of nickel catalyst to pyrazoline. The reduction proceeded more rapidly, and the product gave a dibenzoyl derivative containing a high proportion of the 215–217° isomer. This result indicates that the relative amounts of the two isomers produced in the reduction may vary considerably with the conditions employed. A further study of this point seems desirable.

The properties of the α,γ-diamino acids are those which might be expected. The basic amino acids were obtained as the dihydrochlorides and monohydrochlorides (in one case as the dipicrate). It is perhaps worthy of note that the dihydrochlorides have two disadvantages as characterizing derivatives. They tend to be hydrated and they lose hydrogen chloride (yielding the monohydrochloride) on being heated *in vacuo* or on repeated recrystallization. The latter difficulty can be avoided by recrystallizing the dihydrochloride from glacial acetic acid containing hydrochloric acid. However, with alcohol-ether as the solvent even the addition of small amounts of hydrochloric acid does not entirely prevent loss of hydrogen chloride. The monohydrochlorides of these amino acids are excellent derivatives. They crystallize readily from alcohol or alcohol-ether and show little or no tendency to exist as hydrates.

The Van Slyke amino nitrogen values were theoretical if the reaction time was prolonged to 10 minutes. Dibenzoyl derivatives of the amino acids were readily prepared in good yields.

There is no reason why the reduction of pyrazolines should be limited to those compounds which have a carboxyl group in the 3 or 5 position. Although the preparation of other 1,3-diamines was not investigated, it appears likely that a wide variety of such compounds, which might otherwise be practically inaccessible, could be prepared by this method.

EXPERIMENTAL

DL-α,γ-*Diaminobutyric Acid*—Ethyl pyrazoline-3-carboxylate was prepared in a quantitative yield according to a modification of the method of von Auwers and Cauer (10). A cold solution of 10 gm. (0.1 mole) of ethyl acrylate in 100 ml. of absolute ether was added with shaking to a cold solution of 7.6 gm. (0.19 mole) of diazomethane in 300 ml. of absolute ether. The solution was allowed to stand at 5° for 2 hours with occasional shaking. The excess diazomethane was volatilized at 40° and the resulting colorless solution was concentrated under reduced pressure. The residue was transferred to a filter with the aid of 50 ml. of low boiling petroleum ether. The filtration was conducted rapidly, since the product is unstable in air. The yield of white crystalline material melting at 69–70° was 14.1 gm. This material was used without further purification. A 92 per cent yield of ethyl pyrazoline-3-carboxylate was obtained by the same procedure from only 1 mole of diazomethane per mole of ethyl acrylate.

Ethyl pyrazoline-3-carboxylate (14.1 gm.) was dissolved in alcohol and reduced in the presence of Raney's nickel at 3500 pounds of hydrogen and 65°. The theoretical amount of hydrogen was absorbed in 3 hours. After removal of the catalyst, the combined filtrate and washings were concentrated under reduced pressure to a thick syrup. This was dissolved in 200 ml. of 20 per cent hydrochloric acid and boiled under a reflux for 3 hours. The solution was decolorized and concentrated *in vacuo*. Alcohol (200 ml.) was twice added and removed *in vacuo*. The resulting semisolid mass was treated with 150 ml. of alcohol and 400 ml. of ether. A quantitative yield of the crystalline dihydrochloride was obtained. For recrystallization the dihydrochloride (1.0 gm.) was dissolved in 15 ml. of concentrated hydrochloric acid and 100 ml. of glacial acetic acid were added to the solution. The pure dihydrochloride (0.95 gm.) separated as prisms. This material melted at 175–180° on a micro block[2] and at 200–201° in a capillary. It has previously been obtained by Akabori and Numano (7) who reported a melting point of 202–204°.

For further characterization the dipicrate was prepared by the addition of the calculated amount of picric acid to a hot aqueous solution of the dihydrochloride. On cooling the dipicrate separated as long needles. For analysis α,γ-diaminobutyric acid dipicrate was recrystallized from water, giving material which melted with decomposition at 191–193°.

$C_{16}H_{16}O_{16}N_8$.	Calculated.	C 33.34, H 2.80, N 19.44
(576.4)	Found.	" 33.50, " 2.98, " 19.56

Repeated recrystallization of α,γ-diaminobutyric acid dihydrochloride from alcohol-water mixtures gave the monohydrochloride which melted with decomposition at 246–248°.

$C_4H_{11}N_2O_2Cl$.	Calculated.	C 31.07, H 7.11, N 18.06, NH_2-N 18.06
(154.6)	Found.	" 31.10, " 7.29, " 17.86, " 18.30, 18.00

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[2] The melting and decomposition points reported in this paper were determined on a Köfler micro block unless otherwise specified.

α,γ-Diamino-β-methylbutyric Acid—Methyl 4-methylpyrazoline-5-carboxylate was prepared from methyl crotonate and diazomethane as described for ethyl pyrazoline-3-carboxylate. Von Auwers and Cauer (10) found it necessary to distill the pyrazoline before crystallization could be induced. In our preparation an 88 per cent yield of pure crystalline pyrazoline was obtained directly from the reaction mixture after distillation of the volatile components and cooling the residue in a dry ice-acetone mixture. The product melted at 33–35° (capillary). When not used immediately it was stored under nitrogen at 5°.

Reduction of the pyrazoline and hydrolysis of the reduction product were carried out under the conditions used for α,γ-diaminobutyric acid. The hydrolysate was concentrated to a syrup *in vacuo*. On treatment with absolute alcohol the crystalline α,γ-diamino-β-methylbutyric acid dihydrochloride was obtained in an 82 per cent yield. The material was recrystallized from glacial acetic acid containing hydrochloric acid. The pure product melted at 120–123°.

$C_5H_{14}N_2O_2Cl_2 \cdot H_2O$.	Calculated.	C 26.92, H 7.23, N 12.56
(223.1)	Found.	" 27.16, " 7.24, " 12.68

When heated *in vacuo* or recrystallized from alcohol, the dihydrochloride gradually lost hydrogen chloride. α,γ-Dibenzoylamino-β-methylbutyric acid was obtained in an 82 per cent yield from the dihydrochloride in the usual manner. After being recrystallized from hot water, it melted at 192°. For analysis it was dried *in vacuo* over P_2O_5 for 3 hours at 78°.

$C_{19}H_{20}N_2O_4$.	Calculated.	C 67.04, H 5.92, N 8.23
(340.4)	Found.	" 66.95, " 5.98, " 8.38

Neutral equivalent, 344

α,γ-Diaminoheptanoic Acid—Ethyl 3-*n*-propylpyrazoline-5-carboxylate was prepared from diazobutane and ethyl acrylate. To an ethereal solution of diazobutane (from 33 gm. of N-nitroso-β-butylaminoisobutylmethylketone (11)) at −70° were added 6.5 gm. of ethyl acrylate. The addition was made dropwise, with vigorous shaking, and was stopped as soon as the color of diazobutane disappeared. The reaction was almost instantaneous. The colorless solution was concentrated under reduced pressure (20 mm., 55°) to a colorless oil. This product weighed 11.5 gm. or 96 per cent of the theoretical amount. The material was dissolved in alcohol and reduced immediately.

At an initial hydrogen pressure of 3500 pounds at 70°, the theoretical amount of hydrogen was absorbed in about 2 hours. After removal of the catalyst and evaporation of the alcohol *in vacuo* the remaining syrup was boiled under a reflux for 3 hours with 300 ml. of 20 per cent hydrochloric acid. The hydrolysate was decolorized and concentrated *in vacuo* to a semicrystalline mass. The crude dihydrochloride was dissolved in 200 ml. of warm absolute alcohol and treated with a hot solution of 5 ml. of pyridine in 50 ml. of absolute alcohol. On cooling, the crystalline α,γ-diaminoheptanoic acid monohydrochloride separated. Additional material could be obtained by treating the mother liquors with ether. The yield was 5.6 gm. or 47 per cent of the theoretical amount. On examination of this material on the micro block, it appeared to exist in two crystalline forms, needles (I) and triangular plates (II). The needles appeared to melt at a lower temperature than the plates; the melting point of the mixture was 209–211°.

The separation of the isomeric hydrochlorides was accomplished by fractional crystallization from aqueous alcohol and aqueous alcohol-ether mixtures. The higher melting form was obtained in pure state after several recrystallizations from 85 to 90 per cent alcohol. It consisted entirely of thin triangular plates which melted at 232°, and was designated as hydrochloride (II). After drying at 78° *in vacuo* the following analyses were obtained.

$C_7H_{17}N_2O_2Cl$. Calculated. C 42.75, H 8.72, NH_2-N 14.25
(196.67) Found. " 42.92, " 8.99, " 14.35, 14.52

The second isomer was obtained from the mother liquors of (II) by treatment with absolute alcohol and ether. This material consisted of beautiful bundles of needles which melted at 208–209°. For analysis it was dried for 3 hours at 78° *in vacuo.* It was designated as hydrochloride (I).

$C_7H_{17}N_2O_2Cl$. Calculated. C 42.75, H 8.72, N 14.25, NH_2-N 14.25
(196.67) Found. " 42.96, " 8.85, " 14.07, " 14.37, 14.64

These materials were not interconvertible on crystallization. As further evidence that they were diastereoisomers, the dibenzoyl derivatives were prepared in the usual manner from excess benzoyl chloride in dilute sodium hydroxide.

The dibenzoyl derivative of (I) melted at 174° after being recrystallized from a benzene-methanol-petroleum ether mixture. For analyses it was dried *in vacuo* for 3 hours at 78°.

$C_{21}H_{24}N_2O_4$. Calculated. C 68.46, H 6.57, N 7.61
(368.4) Found. " 68.62, " 6.61, " 7.47
Neutral equivalent, 369

α,γ-Dibenzoylaminoheptanoic acid (II), prepared similarly, melted at 191–193°.

$C_{21}H_{24}N_2O_4$. Calculated. C 68.46, H 6.57, N 7.61
(368.4) Found. " 68.75, " 6.61, " 7.58
Neutral equivalent, 368

α,γ-Diamino-β-phenylbutyric Acid—Ethyl 4-phenylpyrazoline-5-carboxylate was prepared in an 89 per cent yield according to the procedure of von Auwers and Cauer (10). The reduction of the material was carried out under the conditions used for α,γ-diaminoheptanoic acid and was complete in 3 hours. A 70 per cent yield of α,γ-diamino-β-phenylbutyric acid monohydrochloride was obtained by the isolation procedure employed in the above preparation. The material melted with decomposition at 206–210° and appeared to be a mixture of isomers. For analyses it was recrystallized from aqueous alcohol-ether and dried *in vacuo* over P_2O_5 for 3 hours at 78°.

$C_{10}H_{15}N_2O_2Cl$. Calculated. C 52.06, H 6.55, N 12.15, NH_2-N 12.15
(230.7) Found. " 52.02, " 6.90, " 12.28, " 12.56, 12.64

α,γ-Diaminoglutaric Acid—Dimethyl pyrazoline-3,5-dicarboxylate was prepared by Buchner and Papendieck (12) who reported a 52 per cent yield. Using the procedure of these workers we obtained yields consistently 80 per cent or better. 41.5 gm. of dimethyl pyrazoline-3,5-dicarboxylate in 100 ml. of alcohol were reduced at 3500 pounds of hydrogen at 60° in the presence of Raney's nickel. About 50 per cent of the theoretical amount of hydrogen was absorbed in 3 hours. The temperature was raised to 115° and the reduction was complete in an additional 3 hours. After removal of the catalyst the filtrate and washing were concentrated under reduced pressure to a thick blue syrup. The color was probably due to coordination compounds with nickel. The reduction product was hydrolyzed for 3 hours in 300 ml. of 20 per cent hydrochloric acid. After being decolorized, the solution was concentrated *in vacuo* to a thick syrup. In order to remove excess hydrochloric acid, 100 ml. of alcohol were twice added and removed under reduced pressure. The material was then dissolved in 500 ml. of 80 per cent alcohol and 30 ml. of concentrated ammonium hydroxide were added dropwise until the odor of ammonia persisted after thorough shaking. α,γ-Diaminoglutaric acid crystallized as fine needles. After thorough cooling, it was collected on a filter and washed with 100 ml. of cold water, 100 ml. of alcohol, and 100 ml. of ether. The dried product weighed 32 gm. or 88 per cent of the theoretical amount. For recrystallization, the material was dissolved in 3 liters of hot water, the solution was filtered, and to the filtrate were added 3 liters of hot alcohol. On cooling, the amino acid separated as needle-like prisms. The purified material weighed 24.1 gm. and appeared homogeneous. It decomposed above 250° without melting. For analysis the amino acid was dried *in vacuo* over P_2O_5 for 3 hours at 78°.

$C_5H_{10}N_2O_4$. Calculated. C 36.99, H 6.22, N 17.28, NH_2-N 17.28
(162.6) Found. " 36.67, " 6.27, " 17.23, " 17.12, 17.60

α,γ-Dibenzoylaminoglutaric acid was prepared in the usual manner.

After recrystallization from hot water, a 78 per cent yield of pure material, which melted at 206–209°, was obtained. The following analyses were obtained after drying *in vacuo* over P_2O_5 for 3 hours at 78°.

$C_{19}H_{18}N_2O_6$.	Calculated.	C 61.61, H 4.90, N 7.57
(370.35)	Found.	" 61.56, " 4.83, " 7.78

For the preparation of dimethyl α,γ-dibenzoylaminoglutarate, 5 gm. of the acid were suspended in ether and treated with a slight excess of diazomethane in ether. The product (5.2 gm.) melted at 139–141° and gave the correct elementary analyses. It was dissolved in 35 ml. of boiling acetone and 170 ml. of hot hexane (b.p. 60–68°) were added, producing a slight turbidity. After standing at room temperature for 12 hours the precipitate was filtered, washed twice with cold hexane, and dried, giving 2.15 gm. of crystalline rods melting at 151–153°. Recrystallization of this material from acetone (35 ml.) and hexane (70 ml.) gave a pure product melting at 152–153°. For analysis it was dried *in vacuo* over P_2O_5 at 78° for 3.5 hours.

$C_{21}H_{22}N_2O_6$.	Calculated.	C 63.30, H 5.57, N 7.03
(398.41)	Found.	" 63.38, " 5.38, " 7.07

The filtrate from the above separation was taken to dryness. The white solid was dissolved in 20 ml. of hot acetone, and hot hexane (b.p. 60–68°) was added slowly until a slight turbidity was evident (110 ml.). After standing at room temperature for 12 hours a mixture of white crystals separated. The solvent was removed and the residue dried. It was washed three times with 5 ml. portions of cold acetone which removed the 152–153° isomer. From the acetone washings after two recrystallizations was obtained 0.72 gm. of white rods melting at 152°.

The residue from the acetone extraction was recrystallized 3 times by dissolving it in hot acetone and adding hot hexane until the solution became turbid (about 20 volumes). 850 mg. of fine, silky white rods melting at 179–180° were obtained. For analysis the product was dried *in vacuo* over P_2O_5 for 4 hours at 78°.

$C_{21}H_{22}N_2O_6$.	Calculated.	C 63.30, H 5.57, N 7.03
(398.41)	Found.	" 63.42, " 5.65, " 7.07

The filtrates from the above separations, combined and reworked by a similar procedure, yielded 250 mg. of the 152–153° isomer and 300 mg. of the 179–180° isomer. Total recovery of the 152–153° isomer was 3.12 gm. (60 per cent); of the 179–180° isomer, 1.15 gm. (22 per cent).

Diethyl α,γ-dibenzoylaminoglutarate was prepared by dissolving 1.0 gm. of the crude acid in 125 ml. of absolute ethanol to which 0.5 ml. of acetyl chloride had been added. After 4 days at room temperature the solvent was removed under reduced pressure and the residual material crystallized on the addition of a small quantity of water. The crude product after being recrystallized from ethanol-water melted at 124–129°. For analysis it was dried *in vacuo* over P_2O_5 for 3 hours at 60°.

$C_{23}H_{26}N_2O_6$. Calculated. C 64.77, H 6.14, N 6.57
(426.46) Found. " 64.97, " 6.19, " 6.61

This material was separated into two isomers melting at 130–132° and 158–160° respectively by the method employed in separating the methyl esters.

The two methyl α,γ-dibenzoylaminoglutarates were converted to the corresponding acids as follows: 1.0 gm. of the ester melting at 152–153° was treated with 150 ml. of 0.1 N potassium hydroxide in 90 per cent methanol at room temperature for 20 hours. The solution was acidified to pH 3.2 with hydrochloric acid and the methanol removed *in vacuo.* The aqueous solution was then readjusted to pH 1.5 and taken to dryness. Traces of hydrochloric acid were removed in a vacuum desiccator over potassium hydroxide. The solid was recrystallized 3 times from 100 ml. portions of boiling water. 660 mg. of large white needles melting at 203–204° were obtained. The analytical sample was dried *in vacuo* over P_2O_5 for 6 hours at 78°.

$C_{19}H_{18}N_2O_6$. Calculated. C 61.61, H 4.90, N 7.57
(370.35) Found. " 61.70, " 5.11, " 7.83

350 mg. of the isomer melting at 179–180° were treated in a similar manner with 50 ml. of 0.1 N potassium hydroxide in 90 per cent methanol. 280 mg. of white rods were recovered which, after three recrystallizations from 50 ml. portions of boiling water, melted at 215–217°. The analytical sample was dried *in vacuo* over P_2O_5 for 6 hours at 78°.

$C_{19}H_{18}N_2O_6$. Calculated. C 61.61, H 4.90, N 7.57
(370.35) Found. " 61.75, " 4.99, " 7.74

SUMMARY

1. The reduction of pyrazoline-3-carboxylic esters proceeds smoothly, giving excellent yields of α,γ-diamino acids. α,γ-Diaminobutyric acid, α,γ-diamino-β-methylbutyric acid, α,γ-diaminoglutaric acid, α,γ-diamino-β-phenylbutyric acid, and α,γ-diaminoheptanoic acid have been prepared by this method. Two racemic forms of the last three amino acids were produced in the reduction.

The reduction of pyrazolines should afford an easy approach to a wide variety of 1,3-diamino compounds.

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ABSORPTION SPECTRA*

VII. THE INFRA-RED SPECTRA OF SOME NUCLEIC ACIDS, NUCLEOTIDES, AND NUCLEOSIDES

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Nucleoproteins, which seem to be present in viruses, bacteria, animal tissue, and plant tissue and partake in many vital processes (3), are conjugates of various proteins with nucleic acids, and as such may vary in many ways depending on the specific chemical, and perhaps spatial, composition of the whole structure. The brilliant work of Levene (4) and many subsequent investigators (5, 6) has shown that the nucleic acid moieties are polymers of nucleotides (purine or pyrimidine-sugar-phosphates), but little is known concerning the actual structural details.

The two presently recognized types of nucleic acids are that in which the sugar residue is pentose (D-ribose) and that in which the sugar residue is desoxypentose (2-desoxyribose); the first type is exemplified by the nucleic acid which may be prepared from yeast; the second type has been obtained from thymus glands, rat livers, etc. Another difference between the ribose and desoxyribonucleic acids occurs in the pyrimidine portions of the molecules; ribonucleic acid contains cytosine and uracil, whereas the desoxyribonucleic acid contains cytosine and thymine (5-methyluracil) (4), but both materials seem to contain the same purines, namely adenine and guanine. There is also some evidence that the two types of nucleic acids may differ markedly in molecular weight (6, 7), but such work must be closely scrutinized because of the varying methods of extraction and treatment of the materials. The ultraviolet absorption of both types of nucleic acid is essentially similar (8), and it is doubtful whether one can differentiate between them in this region of the spectrum by usual techniques. Our investigation was undertaken with the purpose of attempting to differentiate different types of nucleic acids and their components by means of their infra-red spectra.

It is well known that absorption in the infra-red region of the spectrum is characteristic of particular molecular groupings (9) and relates to the vibrational-rotational motions of the atoms. Thus two closely related substances may be easily differentiated by minor chemical differences, such as the replacement of a hydroxyl group by a hydrogen; even in large molecules and polymers, it is possible to detect small differences in composition (10).

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* For Paper VI, see Blout, Fields, and Karplus (1). A preliminary note on some of the work reported in the present paper has appeared (2). Supported in part by funds from the Office of Naval Research.

The infra-red spectra of ribonucleic acid (from yeast) and desoxyribonucleic (from thymus) are shown in Fig. 1 for the region of 2 to 15 μ (670 to 5000 cm.$^{-1}$). Certain similarities between the two spectra appear obvious; for example, the strong absorptions around 3 μ (which are probably associated with hydroxyl and amino stretching vibrations), the bands at 6 μ (C=C, C=N, and C=O stretching), the bands at 8 μ, and the strong bands at 9.2 μ. The differences between the spectra of the two materials seem to lie mostly at wave-lengths longer than 9 μ; in particular the desoxy compound shows a band at 9.8 μ, not shown in ribonucleic acid in which the 9.2 μ band is symmetrical, and the ribonucleate has a band at 11.7 μ which is not present in the several samples of desoxyribonucleic acid that we have examined.[1] We have not attempted to give any assignment to these absorptions.

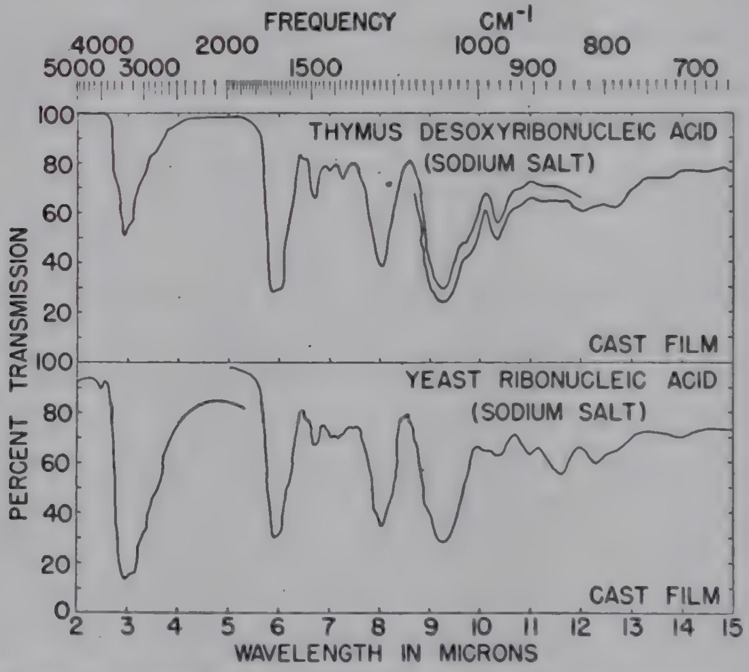

FIG. 1. Infra-red spectra of ribonucleic acid and desoxyribonucleic acid

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[1] It should be noted that because of the very slight solubility of the nucleic acids, nucleotides, and nucleosides in any but aqueous solvents and the rather strong absorption of infra-red by water in the region 2 to 15 μ except in very thin layers it is necessary to measure these materials in the solid state. We have used the following techniques: (*a*) "casting" of a concentrated aqueous solution on silver chloride disks, followed by removal of the water, leaving a continuous film; (*b*) evaporation of the material in high vacuum upon sodium chloride disks; (*c*) finely divided powders on sodium chloride disks; and (*d*) powders mulled into mineral oil. These methods

The spectra of three nucleotides (yeast adenylic,[2] cytidylic, and guanylic acids) have also been determined and are shown in Fig. 2. Guanylic acid gave a satisfactory film upon casting from aqueous solution, but since adenylic and cytidylic acid did not, they were measured as powders, which explains the sloping absorption cut-offs shown by these materials at wavelengths shorter than 5.5 μ. For a comparison of the two techniques we also measured the same sample of guanylic acid as a powder and reproduced its spectral curve in Fig. 2. It is obvious from this and other materials measured in our laboratory that powder films generally give less clearly defined bands than cast or evaporated films, probably due to the scattering of the radiation even at these relatively long wave-lengths (11). These nucleotides are characterized by the strong band or group of bands around 6 μ similar to those seen in the nucleic acids. In addition all the

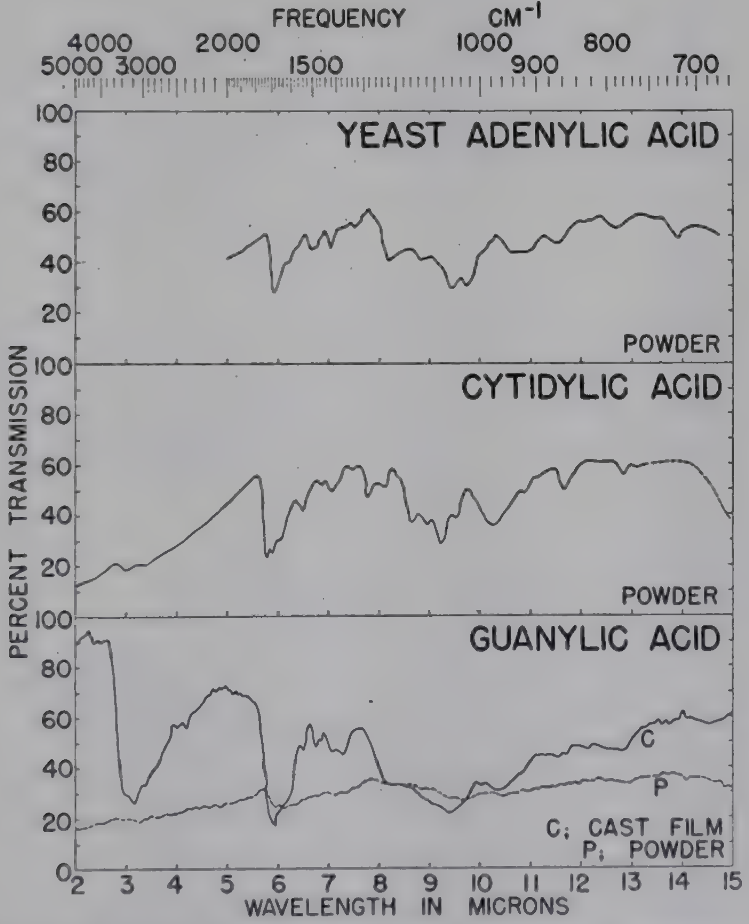

FIG. 2. Infra-red spectra of yeast adenylic, cytidylic, and guanylic acids

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are described in the experimental section and the technique used is noted in the lower right-hand corner of each spectral curve.

[2] Preliminary measurements on a sample of adenosine-5-phosphate (kindly supplied by Dr. Fritz Lipmann) have been made in an attempt to differentiate it from the isomeric 3-phosphate (yeast adenylic acid).

compounds show a band in the region of 9.2 to 9.6 μ.[3] However, from the differences in the spectra of the nucleotides at wave-lengths in the region of 7 to 15 μ, it is obviously possible to differentiate samples of these materials by such measurements.

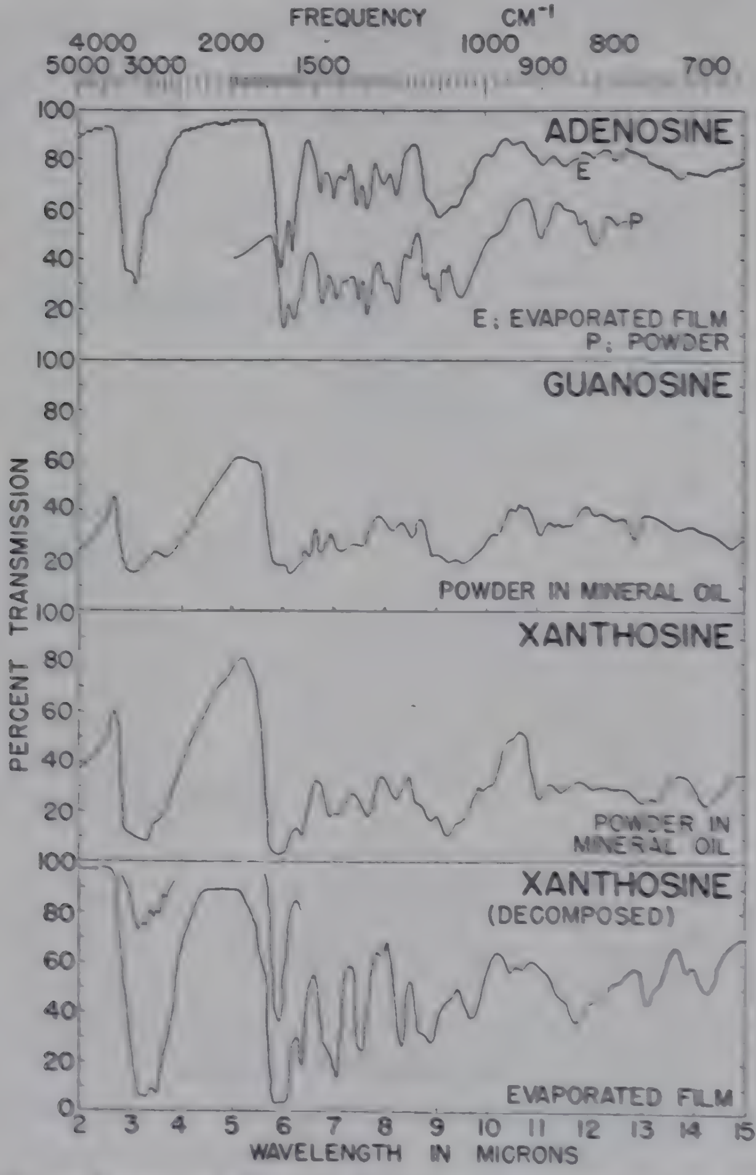

FIG. 3. Infra-red spectra of yeast adenosine, guanosine, and xanthosine

The infra-red spectra of the natural purine ribosides, adenosine and guanosine, as well as the analogous compound, xanthosine, have also been measured and the spectral curves are reproduced in Fig. 3. All the curves are characterized by at least two intense absorption bands in the 3 μ region

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[3] Measurements on too few materials have been made to locate any bands associated with the phosphoric ester portion of the molecules with certainty, although unpublished observations of H. W. Thompson (12) indicate that phosphites and phosphonates absorb in the region of 10.4 to 11.8 μ.

(presumably N—H and O—H stretching) and two intense bands in the 6 μ region (C═C, C═N, or C═O stretching). Numerous other bands (possibly useful for analytical purposes) are observed at longer wave-lengths, including a seemingly characteristic strong absorption in the region 9.2 to 9.6 μ. When adenosine was measured both as an evaporated film and as a powder, good agreement was obtained between the spectra (Fig. 3). Comparison of the spectral curve of xanthosine, as a powder in mineral oil, with that obtained on an evaporated sample indicates, however, that changes in this material must have occurred during the sublimation, since the spectra beyond 8 μ do not agree either in location or intensity of many of the important bands. Perhaps this difference in behavior between adenosine and xanthosine is associated with the free hydroxyl groups on the purine portion of the xanthosine which are not present in adenosine.

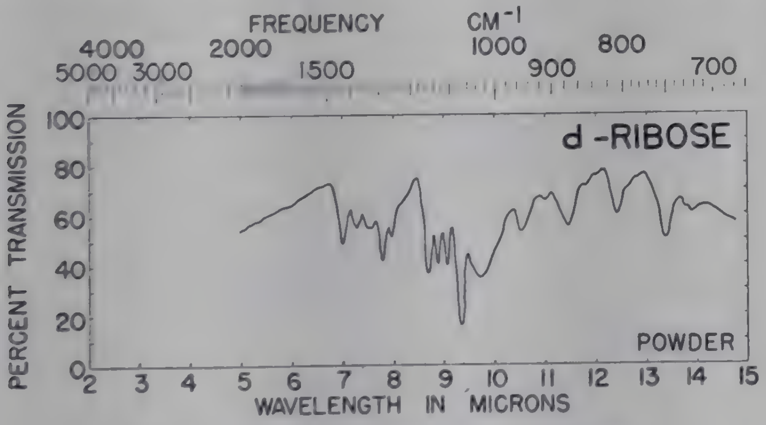

FIG. 4. Infra-red spectra of D-ribose

Finally we have determined the infra-red spectrum of D-ribose (Fig. 4). This material, determined as a powder film, shows very sharp absorption bands beyond 6.5 μ. This is perhaps due to a more uniform particle size distribution because of the crystalline nature of the material. The strongest band in the spectrum is that at 9.3 μ (O—H bending) and seems to be correlatable with strong bands in the 9.2 to 9.6 μ region observed in the more complicated ribose-containing compounds described above.

We wish to acknowledge the assistance of Miss P. L. Snow and Miss A. P. Sutton, who gave invaluable technical aid in this work.

EXPERIMENTAL

Materials[4]—All of the materials were obtained from commercial sources and purified when necessary as shown by ultraviolet absorption measurement or elementary analysis. The data are shown in Table I. The *ribose*

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[4] We are particularly indebted to Dr. Earl D. Stewart of the Schwarz Laboratories for supplying us with samples of some materials not commercially available and with some of the elementary analysis data.

TABLE I

Ultraviolet Spectra and Elementary Analysis of Nucleic Acids, Nucleotides, and Nucleosides

Material	M.p.		Ultraviolet spectral data				Elementary analysis			
	Reported	Found	Reported		Found		Calculated		Found	
			$\lambda_{max.}$	ϵ	$\lambda_{max.}$	ϵ	N	P	N	P
	°C.	°C.	$m\mu$		$m\mu$		*per cent*	*per cent*	*per cent*	*per cent*
Sodium yeast ribonucleate			260*	~32,500	260	32,200	15.30	9.02	14.90	8.84
" thymus desoxyribonucleate†			260	~34,500	252	36,250	15.86‡	9.36‡	14.90	8.79
Adenylic acid	194–195 (4)	195§	260 (14)	14,000	260	13,400	20.18	8.95	20.00	9.10
Cytidylic "	227 (4) 230–233 (4)§	226–228§			272	6,250	13.07	9.62	12.70	9.47
Guanylic "	180 (4)§	174§			252	12,300	19.28	8.54	18.30	8.35
Adenosine	229–230 (4)	225–229	260 (15)	14,500	260	15,700	26.20		25.90	
Guanosine	237 (4)	>250	250 (15)	12,000	250	10,000	24.70		25.00	
Xanthosine		>250	240 (16)	8,600	253	8,790	17.50‖		17.10	

Water was used as a solvent unless otherwise noted. The values for the samples under "Found" were made on samples used for infra-red measurements. Under "Reported" the figures in parentheses refer to the bibliography.

* See Stimson and Reuter (8). Both measurements were made in 0.1 N NaOH.

† The sample from the Dougherty Chemical Company, which was reported to have N 15.3 per cent and P 8.9 per cent, was purified according to the method of Gulland, Jordan, and Threlfall (13).

‡ Calculated for $C_{39}H_{45}N_{15}O_{24}P_4Na_4$, since the Gulland method (see above) was used in purification. The N:P ratio was the same as the calculated (1.69).

§ With decomposition.

‖ With $2H_2O$.

used had a melting point of 82-83° and $[\alpha]_D^{23} = -19.60°$ (reported by Levene and Jacobs (17), m.p. 86–87° and $\alpha_D = -19.5°$).

Preparation of Samples—Four techniques were employed in the preparation of samples for infra-red measurements; *viz.*, (*a*) powder films on sodium chloride disks, (*b*) powders mulled in mineral oil, then spread on sodium chloride disks, (*c*) high vacuum sublimation (at $\sim 10^{-5}$ mm. of Hg) onto sodium chloride disks, and (*d*) casting of aqueous solutions to give continuous films on silver chloride disks. The particular method used for each compound is noted on the absorption curve.

Powder Films (*11*)—To prepare a sample by this method, a small quantity of the compound is ground as finely as possible in an agate mortar; a thin layer of the powder is then spread as evenly as possible over the face of a rock salt disk. A second rock salt disk is then placed on top of the first and rotated slightly so as to produce a more uniform layer. Uniform distribution of the powder over the rock salt disk is facilitated by sifting the material through a fine mesh screen.

Mineral Oil Suspension—When the preparation of satisfactory powder specimens failed, a sample of the material was ground in mineral oil and the suspension placed between rock salt disks for measurement. This technique has the advantage of reducing scattering and of yielding a more uniform sample. On the other hand, the use of mineral oil suffers from the disadvantage that it introduces absorption bands due to the oil. These bands, which occur around 3.4 μ (C—H stretching) and at 6.8 and 7.2 μ (CH_2 and CH_3 deformation), must be corrected for by measuring the sample against an identical cell filled only with mineral oil. This procedure was employed for guanosine and xanthosine.

Vacuum Sublimation—An apparatus was used in which the sample was sublimed (*e.g.*, adenosine sublimed at 170–190° at approximately 10^{-5} mm. of Hg pressure) directly onto a clean rock salt disk held about 3 inches away from the material. In general films obtained by this method are very uniform and often are completely transparent in the visible region. No attempt was made to measure the thickness of the material sublimed, but simply sufficient sublimate was allowed to collect so that the transmission in the 6 μ region was between 10 and 20 per cent. As a check against decomposition during sublimation, samples of a compound before and after sublimation were submitted to spectral measurements in the vicinity of its ultraviolet absorption maximum. In general, the position of the absorption band was unaltered and the ϵ value unchanged (±5 per cent) by this treatment. In some cases, the rate of evaporation was so slow that it was not practicable to collect sufficient material for this purpose.

As noted above (Fig. 3) the spectrum of the evaporated sample of adenosine agreed well with that obtained with a powder of the material between sodium chloride disks. In the case of xanthosine, however, some darkening of the residual material in the sublimer was noted, and on prolonged heating, decomposition occurred. The spectrum of the evaporated sample did indeed agree with that of the mineral oil suspension of xanthosine at wave-lengths shorter than 8 μ, but at longer wave-lengths obvious differences appeared.

Guanosine and adenylic acid charred so readily under conditions required for their sublimation that no attempt was made to evaporate samples of these materials or of the other nucleotides for infra-red measurement.

The simple purines and pyrimidines, on the other hand, showed no apparent evidence of decomposition under the conditions used for sublimation. The infra-red spectral studies on these compounds will be reported shortly.

Cast Films—The technique used can perhaps best be illustrated by the following example. A cast film of sodium ribonucleate was prepared by dissolving 54 mg. of the salt in 0.4 cc. of distilled water and spreading 1 drop of this solution evenly over a 1 inch silver chloride disk. The disk was then placed in a desiccator, stored for at least 24 hours in the dark over phosphorus pentoxide, and then dried for at least 40 hours over this material at room temperature and 1 mm. pressure. The resultant film was continuous, non-crystalline, and satisfactory for infra-red measurements. Films were prepared from thymus desoxyribonucleic acid in a similar manner.

Instrumentation and Measurements—The spectral measurements were made on a Perkin-Elmer infra-red spectrometer, model 12A, with a ten cycle chopper, a Strong bolometer, an alternating current amplifier, and a Brown Instrument Company recording potentiometer. For initial observation, a complete spectrum from 1 to 15 μ was recorded continuously. For drawing the final curves in Figs. 1 to 4, the data were obtained on a point to point basis, the points being taken from 5 to 10 cm.^{-1} apart at frequencies up to 1900 cm.^{-1} (5.2 μ) and at larger intervals at higher frequencies.

SUMMARY

The infra-red spectrum from 2 to 15 μ has been measured for samples of the sodium salts of yeast ribonucleic acid and thymus desoxyribonucleic acid. These compounds exhibit different infra-red spectra, especially at wave-lengths longer than 9 μ.

The infra-red spectra of several nucleotides and nucleosides have also been determined. By use of the infra-red spectra of these compounds, differentiation and identification can be made of pure samples.

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THE DERMAL EXCRETION UNDER CONTROLLED ENVIRONMENTAL CONDITIONS OF NITROGEN AND MINERALS IN HUMAN SUBJECTS, WITH PARTICULAR REFERENCE TO CALCIUM AND IRON*

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Under non-sweating conditions there is a loss of nitrogen and minerals from the skin in the so called "insensible perspiration." Since "insensible perspiration" occurs in all animals, regardless of the presence or absence of sweat glands, the term is not a good one. In man, insensible perspiration is evident to a normal extent in the rare condition known as congenital ectodermal defect (1), in which the sweat glands are totally absent, and in normal persons it persists without diminution when the sweat glands are inactivated by repeated anodal cataphoresis of formaldehyde into the skin (2). Probably insensible perspiration is the result of a passive diffusion of water from the subdermal tissues to the surface of the skin, dependent on a vapor tension gradient. McCance (3) has shown that this diffused fluid, on evaporation at the surface of the skin, deposits there appreciable amounts of nitrogen, potassium, sodium, and chloride. Freyberg and Grant (4) confirmed the presence of these elements in insensible perspiration, together with sulfate sulfur, and in amounts that may be of considerable importance to the accurate determination of mineral balances. Calcium and phosphorus were not found in this excretion.

On the initiation of sweating, the loss of water and solutes in the insensible perspiration presumably stops wherever the surface of the skin is covered with a film of water.

The sensible sweat that supersedes this insensible water (and solute) loss apparently contains all of the minerals found in the blood. Besides sodium, potassium, calcium, magnesium, chlorine, sulfur, and phosphorus, iodine (see (5) and earlier workers) and fluorine (6) have been identified, and concentrations and, in some cases, losses per hour under controlled environmental conditions have been determined. The presence of considerable amounts of bromine in sweat was observed, but not measured, by Spector in the routine of his determinations of iodine in sweat.

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* These experiments were carried out under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

According to Bischoff, Maxwell, and Hill (7), phosphorus does not occur in human sweat. Talbert, Stinchfield, and Staff (8) report variable contents of phosphorus in sweat ranging from 0 to 4.8 mg. per 100 ml. Whitehouse (9) found only doubtful traces of phosphate in sweat.

The magnesium content of sweat is extremely variable in the experience of Hopf (10) and bears no reasonably constant relationship to calcium content, although, on an average, Carpenter and Talbert (11) found a ratio of 4 of calcium to 1 of magnesium, the latter concentration averaging 1.2 mg. per cent. Hopf's average for magnesium in sweat is almost 3 times this figure; namely, 3.32 mg. per cent.

Sweat is generally much more dilute in calcium than blood plasma, though the amounts present may be appreciable from the nutritional point of view. Subject to the usual considerable variation, the following average concentrations of calcium in sweat have been reported in the literature: 0.3 to 1.5 mg. per cent by Marchionini and Ottenstein (12); 5 to 10 mg. per cent by Bryant and Talbert (13); 5.8 mg. per cent by Borchardt (14); 5 mg. per cent by Talbert *et al.* (15); and 4.6 mg. per cent by Hopf (10). The latter investigator observed a marked decrease in the calcium concentration of sweat as sweating proceeds, with initial values as high as 12.2 mg. per cent.

The concentration of nitrogen in sweat has been variously reported from 47 to 130 mg. per cent, and the total output per day from 71 mg. to 5.28 gm., depending upon ambient temperature and especially upon muscular activity. The subject has been well reviewed by Cuthbertson and Guthrie (16). Emotional states, also, will stimulate the secretion of sweat by the apocrine glands and presumably increase the dermal loss of nitrogen and minerals. The nitrogenous compounds of sweat include the main end-products of amino acid metabolism as well as amino acids themselves (17).

A quantitative knowledge of the dermal loss of nitrogen and minerals in the human subject is of importance in three ways: (*a*) It will define the significance of such losses for the accurate determination of nitrogen and mineral balances; (*b*) it will contribute to the better understanding of the body's excretory powers, in iron metabolism in particular; and (*c*) it will, in conjunction with information of compensatory changes in urinary and fecal output, determine whether or not, and to what extent, if at all, nitrogen and mineral requirements are increased under sweating conditions.

The experiments to be reported in the following pages constitute a small part of an extended investigation over a period of 2 years of the loss of water-soluble nutrients in human sweat under controlled environmental conditions. Prior publications in this series deal with the dermal loss of choline (18), of nicotinic acid and its metabolites (19), of folic acid (20), of pyridoxine and its metabolites (21), of inositol and *p*-aminobenzoic acid (22), of ascorbic acid (23), of pantothenic acid (24), of iodine (5), and of fluorine (6). The experiments described below demonstrate the presence of copper, manganese, and iron in sweat, the conditions (food supply and environment) affecting the concentrations of nitrogen, calcium, and iron in sweat and the dermal loss per unit of time. They prove that dermal losses of these elements, even under minimal sweating conditions, constitute an appreciable error in metabolism experiments carried out in the usual way. Under sweating conditions, neglect of these losses may invalidate a metabolism experiment. The skin and sweat glands are shown to be important excretory organs for iron.

TABLE I

Description of Experimental Subjects

Subject	Age	Height	Body weight		Surface area*	Basal metabolism per sq. m. per hr.†
			Initial	Final		
	yrs.	*cm.*	*kg.*	*kg.*	*sq. m.*	*kilocalories*
A	23	167.0	67.0	64.0	1.72	
B	22	163.8	63.0	62.7	1.68	41.7
C	27	165.1	71.3	67.5	1.74	37.8
D	26	187.9	103.4	88.7	2.15	42.0
E	21	172.1	79.4	79.3	1.93	37.6
F	20	163.8	70.6	69.9	1.76	

* Based on final weight, by using the Du Bois and Du Bois formula (25).

† Determined by using the Collins gasometer to collect expired air.

EXPERIMENTAL

Environmental Conditions—The subjects of the experiments were exposed for 7.5 hours daily, 5 days a week, and for 3 hours on Saturday mornings, to controlled environments in an air-conditioned chamber. The environments imposed ranged in sudorific potency from "hot humid" (dry bulb temperature 37–39°, relative humidity 65 to 73 per cent) to "comfortable" (dry bulb temperature 27–28°, relative humidity 43 to 45 per cent). The insensible loss in body weight averaged 720 gm. per hour under hot humid conditions, and 117 gm. per hour under comfortable conditions, with considerable variations from man to man.

Subjects—Six male adult subjects were used in groups of two to four at a time. They were all conscientious objectors on detached service from civilian public service camps. A description of the subjects is given in Table I. While in the experimental chamber, the men generally wore only shorts and klaks, though in the Saturday morning tests, when samples of undiluted sweat were collected, they wore only klaks. In the particular experiments discussed in this paper, the subjects were at rest, sitting in metal chairs except when periodic observations were made on rectal temperatures, or when other occasions required some movement within the chamber.

Dietary Control—The diet was controlled in these particular experiments and was analyzed during metabolism periods when balances of nitrogen, calcium, or iron were being determined. At these times, the diet was made constant throughout a series of 4 weeks and for a preliminary period of 5 or 6 days.

Collection of Sweat—When undiluted sweat was required, generally during the Saturday morning exposures, it was secured either in glass beakers by running the lip of the beaker over selected areas of skin, or in cheese-cloth by periodically wiping the upper ventral part of the body. The sweat thus collected was transferred to glass jars containing acetic acid. Proper precautions were taken to minimize evaporation, and as each jar was filled it was removed to the refrigerator.

Before entering the chamber, each man took a thorough shower bath, using warm water, soap, and a brush. If the sweat was to be used for mineral analysis, the body of each subject was rinsed with doubly distilled water, and the glassware used in collecting and preserving the sweat was cleaned in nitric acid.

When it was desired to obtain the hourly output of nitrogen and minerals in the sweat, the sweat was mopped from the body with preboiled towels or cheese-cloth during the exposure period, and the body, shorts, towels, or cheese-cloth and the chair of each subject were carefully rinsed or washed with distilled water to remove all sweat constituents. Tests of the completeness of collection of sweat by this method, with sweat chlorine as a criterion, indicated that, in washing the body alone, 95 per cent of the chlorine removed in six washings (250 to 300 ml. each) was present in the first four washings. If the second, third, and fourth body washings were used to rinse the towels and shorts of the subjects, which were then rinsed with five more portions of 300 to 500 ml. of distilled water, the chlorine in the last washing was negligible in amount. This procedure was used routinely.

When the iron content of sweat was to be determined, all contact of the body with iron objects was avoided; the metal chairs were covered with rubber sheeting, from which sweat was removed by washing.

The total secretion of sweat was measured by determining the net loss in body weight of each subject during each exposure period. Each subject was weighed to the nearest gm., immediately before entering the chamber and immediately after leaving it, on a Troemner balance, and all food and water consumed during the exposure period, and all urine and feces passed, were similarly weighed. From these measurements, the net loss in body weight was computed.

Obviously, the error in assuming that the net loss in body weight during an exposure period measures the amount of sweat secreted is less the greater the rate of sweating, since in profuse sweating the pulmonary loss of water constitutes an insignificant proportion of the total loss and the insensible perspiration may be considered to have disappeared. Under all sweating conditions the loss in weight due to the respiratory exchange of oxygen and carbon dioxide is insignificant, amounting to only 10.5 gm. per hour for a metabolism of 120 calories per hour and a respiratory quotient of 0.95.

As Lee (26) has pointed out, the content of sweat in a given constituent may be determined more satisfactorily by dividing the total amount removed from the entire body by washing by the net loss in body weight than by analyzing portions of undiluted sweat collected from limited areas of the body surface. This is true because evaporation losses are difficult to avoid in the latter method, but particularly because the composition of sweat from different areas of skin differs.

Analysis of Sweat Samples—All analyses were made upon filtered sweat, which was somewhat opalescent in appearance. The following methods were used, sometimes with slight modifications: nitrogen, Kjeldahl-Gunning-Arnold method and distillation into 4 per cent boric acid; calcium, Larson and Greenberg (27), Kirk and Tompkins (28); magnesium, Briggs (29); phosphorus, Woods and Mellon (30); manganese, Skinner and Peterson (31); iron, Stugart's modification (32) of the method of Kennedy (33); copper, Greenleaf (34).

Results

Minerals and Nitrogen in Undiluted Sweat—Table II contains some analyses of undiluted sweat collected under hot humid conditions. These analyses were carried out in the 1st year of the experiment to determine some of the minerals in sweat and to follow the changes in mineral content that occur during a 3 hour period of sweating. Evidently the copper and manganese contents of sweat are quite small, averaging 5.8 and 6.0 γ per 100 ml., respectively. The phosphorus content is also relatively low, confirming previous work referred to above; the content of calcium, after a period of adjustment, may be considerable, about 1 mg. per 100 ml., while that of magnesium is intermediate. The outputs of calcium and magnesium, per unit volume of sweat, start at relatively high values and then decrease to much lower ones. The phosphorus content shows no consistent change with elapsed sweating time.

Results of some later tests of the changes in the calcium and nitrogen content of sweat as sweating progresses are summarized in Table III. These values confirm the first tests in revealing a sharp drop in the calcium content of sweat. In contrast to calcium (and magnesium), the nitrogen, like the phosphorus, concentration in sweat shows no consistent downward trend with time of sweating.

Paths of Excretion of Nitrogen, Calcium, and Iron under Different Environmental Conditions—The loss of nitrogen, calcium, and iron from the body by way of the alimentary tract, the kidneys, and the skin was measured under profuse sweating conditions (hot humid environment) and under minimal sweating (or non-sweating) conditions (comfortable environment). The essential results are summarized in Tables IV, V, and VI.

Table II

Analyses of Undiluted Sweat, Collected under Hot Humid Conditions, and Effect of Time of Exposure

Date, 1943	Sample No.	Subject A	Subject B	Subject C	Subject D	Average
Copper in γ per 100 ml. sweat						
Feb. 27	1	6.0	6.6	8.0	4.4	
Mar. 13	1	7.5	5.0	4.5	4.5	5.8
Manganese in γ per 100 ml. sweat						
Mar. 6	1	3.2	7.4	7.4	6.0	6.0
Magnesium in mg. per 100 ml. sweat						
July 10	1			0.400	0.286	1st samples, 0.217; 2nd samples, 0.059
" 10	2			0.103	0.057	
" 17	1	0.150	0.188	0.172	0.108	
" 17	2	0.045	0.069	0.075	0.004	
Calcium in mg. per 100 ml. sweat						
May 1	1	4.05	3.22	4.54	3.90	1st samples, 2.94; all others, 0.88
" 1	2	0.73	0.40	0.94	1.63	
" 1	3	0.59	0.71		1.12	
" 1	4	0.64			0.85	
July 3	1	1.31	2.22	2.35	1.90	
" 3	2		0.58	1.21	1.14	
Phosphorus in mg. per 100 ml. sweat						
May 1	1	0.036	0.021	0.043	0.022	
" 1	2	0.012	0.009	0.036	0.015	
" 1	3	0.019	0.032		0.020	
" 1	4	0.028			0.016	0.024

The hourly loss of nitrogen through the skin on a moderately high protein intake (about 98 gm. daily) averaged 152 mg. per hour in a hot humid environment and constituted an average of 22.5 per cent of the total output. Under comfortable conditions the hourly dermal loss of nitrogen averaged 15 mg., accounting for 2.7 per cent of the total output. Even the latter figure assumes an appreciable magnitude for the day; *i.e.*, 360 mg. It is of interest to note that the urinary output of nitrogen during Weeks 24 and 27, when sweating conditions prevailed, was practically the same as the urinary nitrogen during Weeks 25 and 28, when little if any sweating prevailed. These data, therefore, afford no support for the existence of a compensatory relationship between kidney and sweat glands in the elimination of nitrogen, a relationship that seems to exist for chloride, according to Daly and Dill (35). The data, therefore, suggest an increase in nitrogen catabolism under sweating conditions, and hence an increase in protein requirements.

TABLE III

Changes in Mineral Content of Undiluted Sweat with Elapsed Time from Initiation of Sweating

Test 1. Consecutive half hourly samples				Test 2. Consecutive half hourly samples		
Sample No.	Volume of sweat	Ca	P	Sample No.	Volume of sweat	N
	ml.	*mg. per 100 ml.*	*mg. per 100 ml.*		*ml.*	*mg. per 100 ml.*
1	243	5.26	0.075	1	185	45.0
2	437	3.17	0.068	2	518	40.6
3	469	0.96	0.065	3	537	39.6
4	427	0.62	0.045	4	458	30.8
5	389	0.41	0.053	5	427	42.3
6	342	0.40	0.050	6	323	45.4
Averages, Subjects B, C, D, and E; chamber conditions, dry bulb 37°, wet bulb 33°, relative humidity 76%				Averages, Subjects C, D, and E; chamber conditions, dry bulb 38°, wet bulb 32°, relative humidity 65%		

Comparison of the metabolism of calcium in a hot and a comfortable environment (Table V) is complicated by the greater calcium intake in the former environment. This was brought about by a greater consumption of water, which unfortunately was tap water, containing about 60 mg. of calcium per liter. Under profuse sweating there was an average cutaneous loss of 20.2 mg. of calcium per hour, compared with a loss of 6.2 mg. under comfortable conditions. The former loss accounted on the average for 29.9 per cent of the total body loss of calcium, over twice that in the urine. Even under non-sweating conditions, 14.4 per cent of the total output of calcium occurred, on the average, through the skin, not greatly different from the proportionate loss in the urine, 21.0 per cent. There is little suggestion from the data in Table V that an increased excretion of calcium through the sweat glands is associated with a decreased excretion in the urine, though the difference in calcium intake in the two environments does not permit an unequivocal answer to this problem.

TABLE IV

Paths of Excretion of Nitrogen under Different Experimental Environments

Subject	Experimental wk.	Net loss in body weight per hr.	Daily intake of N	Hourly loss of N			Loss of N*		
				From alimentary tract†	Through kidneys†	Through skin‡	From alimentary tract†	Through kidneys†	Through skin‡
		gm.	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
C	24th	498	15.7	57	418	128	9.5	69.3	21.1
D	24th	738	20.1	74	560	146	9.5	71.7	18.7
E	24th	680	17.2	77	462	145	11.3	67.5	21.2
F	24th	438	14.9	54	424	127	9.0	70.0	21.0
C	25th	86	13.0	69	408	19	14.0	82.2	3.8
D	25th	126	16.2	90	526	21	14.1	82.5	3.3
E	25th	66	14.3	62	467	10	11.6	86.6	1.8
F	25th	54	12.6	54	432	6	10.9	87.8	1.3
C	27th	635	14.3	58	413	180	8.8	63.5	27.6
D	27th	863	19.1	64	553	178	8.0	69.6	22.3
E	27th	969	16.0	60	423	214	8.7	60.6	30.7
F	27th	410	13.8	52	426	101	9.0	73.5	17.5
C	28th	79	14.9	52	440	19	10.1	86.2	3.7
D	28th	132	19.0	73	562	20	11.2	85.8	3.0
E	28th	80	16.2	62	431	14	12.2	85.1	2.7
F	28th	51	14.4	72	394	9	15.2	83.0	1.8
Average									
Wks. 24 + 27§..		654	16.4	62.0	460	152	9.2	68.2	22.5
" 25 + 28‖..		84	15.1	66.8	458	15	12.4	84.9	2.7

* Computed on total excretion.

† Average for 5 full days.

‡ Average for five daily exposures to the experimental environment of 7.5 hours each.

§ Hot humid environment.

‖ Comfortable environment.

The data summarized in Table VI are the only evidence available, in so far as the authors are aware, of the output of iron in human sweat. In the particular experimental weeks covered in Table VI, the iron intake was deliberately made excessive by the administration to the subjects of a daily supplement of ferric chloride containing 25 mg. of iron. The output of iron per hour averaged 1.29 mg. in the hot environment and 0.27 mg. in the comfortable environment, both values exceeding the urinary output by many times. On a percentage basis of the total output, the values were, respectively, 37.2 and 13.4. Although the amounts of iron excreted through the kidneys per day were generally less under sweating than under non-sweating conditions, the urinary iron is so small in amount under all conditions that this compensatory relationship, if real, is insignificant in magnitude.

TABLE V

Paths of Excretion of Calcium under Different Experimental Environments

Subject	Experimental wk.	Net loss in body weight per hr.	Daily intake of Ca	Hourly loss of Ca			Loss of Ca*		
				From alimentary tract†	Through kidneys†	Through skin‡	From alimentary tract†	Through kidneys†	Through skin‡
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
C	32	77	744	22	8.7	5.9	60.3	23.6	16.0
D	32	135	1053	35	11.8	7.4	64.7	21.6	13.6
E	32	75	737	29	7.5	5.0	69.8	18.1	12.0
F	32	61	819	23	6.1	2.9	71.4	19.4	9.2
C	33	692	997	32	9.7	18.0	53.9	16.1	30.0
D	33	861	1476	49	10.2	22.2	60.3	12.5	27.2
E	33	816	1090	37	8.3	23.8	53.6	12.0	34.4
F	33	607	1046	34	4.8	12.5	66.4	9.3	24.3
C	34	86	756	26	10.1	8.0	59.4	22.6	17.9
D	34	157	1118	32	12.4	9.6	59.0	23.1	17.9
E	34	69	778	27	8.5	6.1	65.2	20.3	14.6
F	34	63	846	22	6.4	4.6	66.6	19.4	14.0
C	35	710	951	27	10.7	17.5	48.8	19.4	31.8
D	35	934	1430	52	11.2	23.8	60.0	12.8	27.2
E	35	832	1071	42	9.8	27.2	53.3	12.4	34.3
F	35	704	1091	37	1.6	16.2	67.3	2.9	29.7
Averages									
Wks. 33 + 35§..		770	1144	38.8	8.3	20.2	58.0	12.2	29.9
" 32 + 34‖..		90	856	27.0	8.9	6.2	64.6	21.0	14.4

* Computed on total excretion.

† Averages for 5 full days.

‡ Averages for five daily exposures to the experimental environment of 7.5 hours each.

§ Hot humid environment.

‖ Comfortable environment.

Concentration of Nitrogen, Calcium, and Iron in Total Sweat Secreted and Effect of Level of Intake—By taking the net loss in body weight as a measure of total sweat loss and dividing it into the amounts of the elements removed from the body by washing, both measurements extending over a period of

7.5 hours for 5 days a week (except as indicated), it is possible to estimate the concentration of the elements in total body sweat, or total insensible perspiration when sweating does not occur. The accuracy of this calculation has been discussed above. It avoids the error inherent in the method of taking undiluted, but possibly not unconcentrated, sweat from restricted areas of the body, due to the variable rates of secretion (36, 37) and variable sweat composition from different parts of the skin surface (38). Estimations of sweat composition made in this way, in different environments and for different intake levels of nitrogen, calcium, and iron, are summarized in Tables VII, VIII, and IX.

TABLE VI

Paths of Excretion of Iron under Different Experimental Environments

Subject	Experimental wk.	Net loss in body weight per hr.	Daily intake of iron	Hourly loss of iron			Loss of iron*		
				From alimentary tract†	Through kidneys†	Through skin‡	From alimentary tract†	Through kidneys†	Through skin‡
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
C	17	653	50.8	2.39	0.012	1.68	58.5	0.3	41.2
D	17	879	76.0	2.41	0.023	1.93	55.3	0.5	44.2
E	17	909	68.3	2.12	0.022	1.72	54.8	0.6	44.6
C	18	101	43.2	1.81	0.051	0.17	89.0	2.5	8.4
D	18	156	54.6	1.61	0.038	0.29	83.1	2.0	15.0
E	18	82	47.8	1.49	0.066	0.16	86.7	3.8	9.4
C	19	103	45.8	1.55	0.025	0.53	73.8	1.2	25.0
D	19	132	52.5	1.90	0.031	0.32	84.4	1.4	14.2
E	19	73	46.2	1.53	0.039	0.14	89.5	2.3	8.2
C	20	501	46.9	1.84	0.009	0.56	76.4	0.4	23.2
D	20	764	56.9	2.03	0.035	0.83	70.1	1.2	28.7
E	20	798	49.8	1.36	0.026	0.99	57.2	1.1	41.8
Averages									
Wks. 17 + 20§		751	58.1	2.02	0.021	1.29	62.0	0.7	37.2
" 18 + 19‖		108	48.4	1.65	0.042	0.27	84.4	2.2	13.4

* Computed on total excretion.

† Average for 5 full days.

‡ Average for five daily exposures to the experimental environment of 7.5 hours each.

§ Hot humid environment.

‖ Comfortable environment.

The computations in Table VII reveal an increasing nitrogen content of the sweat with an increase in nitrogen intake of about 100 per cent, judging from the averages alone. This finding agrees with those of Cuthbertson and Guthrie (16). However, the average differences in the nitrogen con-

TABLE VII

Effect of Rate of Sweating and Nitrogen Intake on Dermal Loss of Nitrogen and Nitrogen Concentration in Sweat

Subject	Experimental wks. averaged	Net loss in body weight per hr.	Intake of N per day	Dermal loss of N	
				Per hr.	Per kilo of sweat*
		gm.	*gm.*	*mg.*	*mg.*
C	32 + 34	82	11.06	17.7	216
D	32 + 34	146	16.24	22.7	155
E	32 + 34	72	11.47	11.0	153
F	32 + 34	62	13.28	8.5	137
C	33 + 35	701	11.02	194	277
D	33 + 35	897	16.34	178	198
E	33 + 35	824	11.05	167	203
F	33 + 35	655	13.26	151	231
Average.....	32 + 34†	91	13.01	15.0	165
	33 + 35‡	769	12.92	173	227
C	25 + 28	82	13.94	18.6	227
D	25 + 28	129	17.62	20.4	158
E	25 + 28	73	15.26	12.0	164
F	25 + 28	53	13.47	7.2	136
C	24 + 27	566	15.01	154	272
D	24 + 27	800	19.59	162	203
E	24 + 27	824	16.64	180	218
F	24 + 27	424	14.35	115	271
Average.....	25 + 28†	84	15.07	14.6	171
	24 + 27‡	654	16.40	153	241
C	18	101	18.39	21.7	215
D	18	156	24.46	23.6	151
E	18	82	20.29	12.8	156
C	17	653	21.25	196	300
D	17	879	26.29	219	249
E	17	909	22.47	226	249
Average.....	18†	113	21.05	19.4	174
	17‡	814	23.34	214	266

* Taking the net loss in body weight as a measure of sweat secretion or insensible perspiration.

† Dry bulb 29°, wet bulb 22°, relative humidity 50 to 52 per cent.

‡ Dry bulb 38°, wet bulb 33°, relative humidity 68 per cent.

tent of sweat were not great over this range of nitrogen intake, being from 165 to 174 mg. per liter under comfortable conditions, and from 227 to 266 mg. per liter under hot humid conditions. Furthermore, a consideration of the performance of individual subjects at the three levels of nitrogen intake detracts considerably from the evidence supporting a positive correlation. The conclusion that no evidence approaching certainty has been secured for an effect of nitrogen intake on the nitrogen content of sweat, or on the dermal loss of nitrogen, is in harmony with the findings of Berry (39).

TABLE VIII

Effect of Rate of Sweating and Calcium Intake on Dermal Loss of Calcium and Calcium Concentration in Sweat

Subject	Experimental wks. averaged	Net loss in body weight per hr.	Intake of Ca per day*	Dermal loss of Ca	
				Per hr.	Per kilo of sweat†
		gm.	*mg.*	*mg.*	*mg.*
C	32 + 34	82	750	6.89	84
D	32 + 34	146	1085	8.55	59
E	32 + 34	72	757	5.58	78
F	32 + 34	62	833	3.76	61
C	33 + 35	701	974	17.8	25
D	33 + 35	897	1453	23.0	26
E	33 + 35	824	1080	25.6	31
F	33 + 35	655	1068	14.5	22
Average.....	32 + 34‡	91	856	6.20	70.5
	33 + 35§	769	1144	20.2	26.0
C	18 + 19	102	2888	4.61	45
D	18 + 19	144	2963	4.62	32
E	18 + 19	77	2952	3.95	51
C	17 + 20	577	2969	9.55	17
D	17 + 20	821	3200	9.97	12
E	17 + 20	853	3142	10.40	12
Average.....	18 + 19‡	108	2934	4.39	42.7
	17 + 20§	750	3104	9.97	13.7

* In Weeks 17 to 20, inclusive, the calcium intake was raised over the usual level, as in Weeks 32 to 35, inclusive, by the addition of milk (500 ml. per day) and of 1.1 gm. of calcium as dicalcium phosphate.

† Taking the net loss in body weight as a measure of sweat secretion or insensible perspiration.

‡ Dry bulb 29°, wet bulb 22°, relative humidity 50 to 52 per cent.

§ Dry bulb 38°, wet bulb 33°, relative humidity 68 to 69 per cent.

The lower estimated concentration of nitrogen in the dermal secretions under comfortable as compared to hot humid conditions is clear cut. It may mean either (*a*) that the nitrogen content of sweat is directly correlated with the rate of sweat secretion, or (*b*) that the dermal loss of nitrogen under

TABLE IX

Effect of Rate of Sweating and Iron Intake on Dermal Loss of Iron and Iron Concentration in Sweat

Subject	Experimental wks. averaged	Net loss in body weight per hr.	Intake of iron per day	Dermal loss of iron	
				Per hr.	Per kilo sweat*
		gm.	mg.	mg.	mg.
B	9	115	No Fe supplement	0.268	2.33
C	9	135	“ “ “	0.209	1.55
D	9	165	“ “ “	0.350	2.12
E	9	121	“ “ “	0.199	1.64
B	10	115	“ “ “	0.212	1.84
C	10	135	“ “ “	0.117	0.87
D	10	432	“ “ “	0.412	0.95
E	10	183	“ “ “	0.402	2.10
Average..........	9†	134	“ “ “	0.257	1.91
	10‡	216	“ “ “	0.286	1.44
B	13	402	No Fe supplement	0.957	2.38
C	13	534	“ “ “	1.203	2.25
D	13	753	“ “ “	0.632	0.84
E	13	636	“ “ “	0.880	1.38
B	14	65	“ “ “	0.134	2.06
C	14	86	“ “ “	0.170	1.98
D	14	118	“ “ “	0.228	1.93
E	14	80	“ “ “	0.178	2.23
Average..........	13§	581	“ “ “	0.918	1.71
	14‖	87	“ “ “	0.178	2.05
C	18‖	101	43¶	0.171	1.69
D	18‖	156	55¶	0.290	1.86
E	18‖	82	48¶	0.163	1.99
C	19‖	103	46¶	0.528	5.13
D	19‖	132	53¶	0.321	2.43
E	19‖	73	46¶	0.141	1.93
C	17§	653	51¶	1.68	2.57
D	17§	879	76¶	1.92	2.18
E	17§	909	68¶	1.72	1.89
C	20§	501	47¶	0.563	1.12
D	20§	764	57¶	0.833	1.09
E	20§	798	50¶	0.998	1.25

* Taking the net loss in body weight as a measure of sweat secretion or insensible perspiration.

† Dry bulb 32°, wet bulb 27°, relative humidity 66 per cent.

‡ Dry bulb 32.2°, wet bulb 31.7°, relative humidity 95 per cent. These values are averages of 2 instead of 5 days.

§ Dry bulb 38.3°, wet bulb 32.8°, relative humidity 67 to 70 per cent.

‖ Dry bulb 29.4°, wet bulb 21.7°, relative humidity 50 per cent.

¶ Including a supplement of 25 mg. of iron daily as $FeCl_3$.

comfortable conditions is largely brought about by the insensible perspiration, which may be lower in nitrogen than sensible perspiration. Also, the net loss in body weight includes the loss of moisture from the respiratory tract, which would be nitrogen-free. This fraction of the net loss in body weight, while insignificant under conditions of profuse sweating, may amount to 30 per cent under non-sweating conditions (1).

Similar data with reference to calcium are presented in Table VIII. In these experimental weeks, the hourly loss of calcium through the skin, as well as the estimated concentration of calcium in the sweat, was considerably higher on the low calcium than on the high calcium régime. This is an anomalous result, the explanation for which is not evident. In both series of weeks, the estimated calcium content of the "sweat" produced under fairly comfortable conditions is almost 3 times as great as the estimated calcium content of the sweat produced in a hot humid environment. This is contrasted with the analogous situation with reference to nitrogen (Table VII), and is unexpected on the basis of the reported absence of calcium from the insensible perspiration (4). The obvious interpretation of these results is that the concentration of calcium in sweat is inversely related to the rate of sweating.

For 8 weeks of the experiment, the losses of iron through the skin were determined by analyzing the body washings. During Weeks 17 to 20, inclusive, the three subjects then available were undergoing a metabolism study involving analysis of the food, feces, and urine. The iron intake was high because of a daily supplement of ferric chloride providing 25 mg. of this element. The subjects were on constant diet during Weeks 9, 10, 13, and 14, but the intake of iron was not measured, though presumably it was approximately half the intake of Weeks 17 to 20. From the values summarized in Table IX, it appears that the concentration of iron in sweat averages about 2 mg. per liter, with considerable variations on either side of this value. The evidence presented in Table IX does not establish any clear cut effect of the range in iron intake, or in environmental conditions, on the iron content of sweat. It does indicate, however, that the skin is an important path of excretion of iron, far more important than the kidneys (Table VI), even under environmental conditions inducing minimal activity of the sweat glands (Weeks 14, 18, and 19).

DISCUSSION

Under comfortable conditions, in which sweating is minimal, the subjects of these experiments excreted per hour through the skin an average of 15 mg. of nitrogen, 6.2 mg. of calcium, and 0.27 mg. of iron. These values indicate the order of magnitude of the error incurred in balance experiments involving these elements by neglecting dermal losses. On the 24 hour basis these values become, respectively, 360, 149, and 6.5 mg.; the values for calcium and iron in particular represent considerable errors, compared with requirements. When general sweating is initiated in response to a thermal stimulus, these dermal losses were increased from 3.3 to 10 times under hot humid conditions, under which total sweating averages 700 to 800 gm. per hour. Evidently, satisfactorily accurate balance experiments with reference to nitrogen, calcium, and iron cannot be carried out under sweating conditions, either emotional or thermogenic, unless dermal losses are measured. A fairly satisfactory method of estimating these losses may possibly be based upon a determination of net loss in body weight and a determination of the concentration of the element in a sample of hand sweat. Johnston, Conn, Louis, and Steele (40) claim that the daily concentrations of chloride and nitrogen in hand sweat represent reasonably accurately their concentrations in total body sweat. This work needs confirmation, and extension to calcium and iron.

The capacity of the body to excrete iron is seriously restricted according to the newest theory of iron metabolism, which has been well stated by Johnston (41). In this theory it is claimed that excretion of iron through the kidney and the gut is almost insignificant compared with usual iron intakes. Hence, the body absorbs only as much iron as it needs and uses that amount over and over again, with little loss. This peculiar theory considers excretion possible only through the kidneys and the gut. The findings of these experiments that the skin and the sweat glands are important excretory organs for iron render unnecessary the assumption of physiological mechanisms of unknown nature (or doubtful efficacy) for limiting iron absorption and for getting rid of iron absorbed in excess of needs. The high content of iron in hair, 0.013 to 0.017 per cent, according to Bagchi and Ganguly (42) using carefully washed samples, represents another appreciable outlet of iron from the body.

Whether or not the function of the skin and of the sweat glands in excreting nitrogen and minerals raises the body's requirements for these elements depends upon possible compensatory relationships between the losses through the skin and those through the kidneys and the alimentary tract. The experiments reported above offer no support for such relationships, but the conditions for detecting them were not ideal. If no such relationship exists for nitrogen, calcium, and iron, as it does for chloride, then dermal losses will increase bodily requirements in direct proportion to the losses themselves. The subject is of sufficient importance to warrant further study.

SUMMARY

1. The dermal losses of nitrogen, calcium, iron, magnesium, phosphorus, copper, and manganese were studied with six adult human subjects subjected to controlled environmental conditions.

2. Human sweat contains small amounts of copper and manganese, about 6 γ per 100 ml., moderate amounts of magnesium and phosphorus, 0.22 to 0.022 mg. per 100 ml., and relatively large amounts of iron, 1 to 2 mg. per liter, of nitrogen, 170 to 245 mg. per liter, and calcium, 20 to 70 mg. per liter.

3. The concentration of calcium and magnesium in sweat decreases as sweating progresses, while that of nitrogen and phosphorus remains essentially unchanged.

4. The concentration of nitrogen and iron in sweat has not been found to be dependent on the intake of these elements; that of calcium was anomalously decreased on raising the intake of this nutrient from usual to excessive levels.

5. Under conditions of profuse, as compared with minimal, sweating, the concentration of nitrogen in sweat increases, that of calcium decreases, and that of iron is unaffected.

6. The dermal losses of nitrogen, calcium, and iron under minimal sweating conditions, computed on the daily basis, averaged 360, 149, and 6.5 mg., respectively. Under profuse sweating conditions, these losses may increase from three to ten times, depending on the element.

7. The skin and the sweat glands are important excretory organs for iron.

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ANTIRACHITIC SULFONATION OF SOME STEROIDS*

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A few sterols, some derivatives, and certain steroid materials have been transformed chemically, by heating with suitable reagents (1–4), to products having antirachitic activity. This activating reaction[1] involves heating an appropriate substrate in acetic acid solution with an efficient sulfonating reagent under anhydrous conditions for a number of hours. At the termination of the reaction time, the acetic acid is removed by vacuum distillation. The antirachitic material is contained in the residue. Among the various sulfonating reagents tried in acetic acid solution, sulfuric acid-acetic anhydride or chlorosulfonic acid generally has produced residues having the greatest potencies when the substrate was either cholesterol or those derivatives of it which are unsaturated at carbon 5 and react positively to the Liebermann-Burchard blue to green color test (5). Since the appearance of the earlier reports referred to above, the procedure of antirachitic sulfonation has been variously modified and applied to some of the oxidized cholesterol derivatives in addition to cholesterol and to certain steroid-containing materials. This communication presents the results of some of these studies.

EXPERIMENTAL

Antirachitic Sulfonation of Various Sterols—The general procedure of antirachitic sulfonation has been applied with varying degrees of effectiveness to various sterols. Some of the results are presented in Table I. Here it will be seen that the antirachitic potency[2] of the sulfonated end-product depends in part on the nature of the steroid substrate as previously reported (4). Antirachitic sulfonation of isocholesterol produced a residue containing no antirachitic activity (Experiment 34). This isocholesterol was separated from the unsaponifiable fraction of wool fat by the method of Doree and Garratt (8) and thus was composed entirely of lanosterol and a

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[1] This reaction will be referred to as "antirachitic sulfonation" and the antirachitic products formed by the reaction as "chemosterol D."

[2] The biological tests were conducted in accordance with the line test procedure of U. S. P. XI (6) with exceptions as to numbers of rats indicated in the tables and their grouping.

Table I

Antirachitic Sulfonation of Steroid Materials

Experiment No.	Steroid material	Weight	Purity*	Reagents and amount used					No. of rats per group	Steroid ≎ fed per rat	Average line test response plus values†
				Kind	Amount	AcOH	Ac_2O	Temperature			
		gm.	*per cent*		*ml.*	*ml.*	*ml.*	°*C.*		*mg.*	
91‡	Cholesterol	3.86	C.P.	H_2SO_4	1.12	35	3.3	85	8	6	2.0
79	"	1.93	" "	"	3 × 0.3	20	3 × 0.8	85	4	3	2.0
299	"	10	85	" SO_3, 20%	3.0	80	6	85	3	5.8	2.0
									3	11.6	2.0
305	"	100	85	"	37	800	70	85	3	6	2.0
									3	10	2.5
309	"	10	85	"	2.8	50	10	110	3	4	1.0
									3	8	1.5
310	"	10	85	"	2.8	50	10	125	3	4	1.0
									3	8	2.5
319	"	10	75	"	2.5	50	7.5	95	3	6	2.0
									3	12	2.5
337	"	3.86	C.P.	"	1.1	0	5	125	3	6	1.5
									3	12	2.7
385	"	80	" "	"	22	800	80	117	3	3	2.1
									3	6	2.5
297‡	Cholesteryl acetate	0.44	" "	"	0.13	3	0.5	85	6	3	0.0
									6	12	2.0
510	" chloride	1.62	" "	HSO_3Cl	0.52	30	0.4	117	4	7.5	2.5
									4	75	5.0
339	Cholesterol HCl	0.42	" "	H_2SO_4	0.11	4	0.4	85	3	6	1.0
									3	12	2.0
		0.42	" "	"	0	4	0.4	85	3	12	0

64	Corn germ sterol	0.4		H_2SO_4	0.12	4.5	0.5	85	4	60	3.0
54	Sperm oil "	0.6		"	0.16	6	0.45	85	4	600	0
34	Isocholesterol	2.1		"	0.6	35	4	85	4	150	0
105	Lanosterol	1.0		"	0.26	9	0.55	85	4	250	0
106	" dehydrated	1.0		"	0.26	9	0.55	85	4	250	0

* Based upon the gravimetric digitonin assay procedure (7).

† Negative control tests were conducted routinely for all experiments. No rats showed healing. Complete healing is indicated by 5.0 (6).

‡ Composites of two replicates.

small percentage of agnosterol. Windaus and Tschesche (9) separated isocholesterol of wool fat into 92 per cent lanosterol and 8 per cent agnosterol. The negative results given by sulfonated isocholesterol had been anticipated, since lanosterol does not give the Liebermann-Burchard color reaction of the steroids unsaturated at carbon 5, and in view of later reports (10–12) neither lanosterol nor agnosterol can be considered true sterols.

Earlier tests (1) indicated that, when cholesterol was dehydrated to cholesterilene, the latter produced a more potent antirachitic when sulfonated. Therefore, pure lanosterol, 3 gm., prepared by the Doree and Garratt (8) procedure, was dehydrated by first heating in an oil bath to 200° and then for 10 minutes with 0.5 gm. of powdered fused zinc chloride. This caused an evolution of gas. The reaction product was extracted with petroleum ether, filtered free from zinc chloride, and the solvent removed by evaporation at 100°. 1 gm. of the oily residue was sulfonated exactly as lanosterol had been in Experiment 105 and tested subsequently for antirachitic activity (Experiment 106). Again no antirachitic was produced. Therefore, it appears that cholesterol is the only known sterol in wool fat which can be antirachitically sulfonated.

Sulfonated sperm oil sterol, Difco[3] (13), also yielded products having no antirachitic activity, even when fed at comparatively large levels of intake. Furthermore, sulfonated ergosterol, stigmasterol, and β-sitosterol (4) yielded products having relatively little antirachitic potency. On the other hand, when the same activating procedure was applied to the mixed sitosterols of corn germ sterol, Difco, an active residue with much higher potency was obtained.

Steps also were taken to determine what modification in procedure might be effective in increasing the antirachitic potency of chemosterol D derived from various grades of cholesterol, its acetate, chloride, and hydrochloride. Many variations in the proportion of the sulfonating reagents and conditions of operation were tried. The results of some of these also are reported in Table I (Experiments 91, 305, 309, 310, 319, and 385). These tests demonstrate that either pure or crude cholesterol preparations may be used as the substrate, and that the composition of the reacting mixture may vary considerably without affecting appreciably the antirachitic potency of the sulfonated end-product. Also, the effect of successive treatments of substrate with the same sulfonating reagents was tried (Experiment 79). In this instance, the successive reheating of 1.93 gm. of cholesterol with two fresh portions of the sulfuric acid-acetic anhydride mixture gave a residue which appeared to have a greater potency than the same amount of cholesterol treated only once with the same sulfonating mixture. Also interesting is the observation that the presence of hydrogen chloride in the cholesterol addition product did not prevent antirachitic sulfonation (Experiment 339); nor did substitution of chlorine for the 3-hydroxyl group of cholesterol in the cholesteryl chloride (Experiment 510) reduce appreciably the antirachitic potency usually produced from similar quantities of cholesterol.

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[3] A commercial preparation kindly supplied by the Difco Laboratories, Inc., Detroit, Michigan.

Exploratory tests (Experiments 309, 310, 319, 337, and 385) had indicated that sulfonation at higher temperatures than employed originally might favor the production of residues having greater potencies. Subsequently, experiments were undertaken to determine whether sulfonation of cholesterol at higher temperatures and longer periods of heating would produce chemosterol D having greater biological activity. The results obtained are summarized in Table II. Here, the general sulfonation procedure involved the gradual addition of 11 ml. of sulfuric acid (specific gravity 1.84) to an agitated suspension of 40 gm. of cholesterol in 400 ml. of 99.5 per cent acetic acid and 40 ml. of acetic anhydride. At the end of each heating period, the acetic acid was removed by distilling in partial vacuum from a boiling water bath. Biological tests of the distillation residues indicate that, when the heating period was limited to 3 hours, greater antirachitic activity was obtained by carrying out the reaction at 117°, the temperature of boiling acetic acid, rather than at 75°. Furthermore, extending the reaction time to 16 hours at either of these temperatures failed to increase appreciably the antirachitic potency of the residue.

TABLE II

Effect of Length of Time and Temperature of Reaction on Antirachitic Sulfonation of Cholesterol

Experiment No.	Reaction time	Temperature	Cholesterol for 1 unit (U. S. P.) of activity
	hrs.	°*C.*	*mg.*
368	0.5	97	4.50
369	3	97	0.85
370	8	97	0.89
371	16	97	0.68
372	24	97	0.69
373	3	75	1.33
374	3	117	0.35
375	16	75	0.47
376	16	117	0.30

Antirachitic Sulfonation of Oxidized Cholesterol Derivatives—Antirachitic sulfonation of sterols requires, in addition to other reagents, the use of sulfuric acid or its derivative chlorosulfonic acid (14). The anhydrous acid apparently oxidizes the organic substrate with the liberation of sulfur dioxide (3). Since antirachitic sulfonation is potentially an oxidizing reaction, several oxidized cholesterol derivatives were prepared and sulfonated. The results are presented in Table III. The prepared oxidized derivatives of cholesterol listed here were heated 14 hours in an acetic acid solution to which had been added minimum amounts of acetic anhydride and sulfuric acid, or chlorosulfonic acid. These acids were added in the amounts indicated with stirring at room temperature and then heated. At the termination of the period of heating the acetic acid was distilled at 55° in a partial vacuum. Then the residue was dissolved in a mixture of ethylene chloride and methanol, brought to volume, and subsequently tested biologically for antirachitic activity.

These oxidized steroids may be considered derivatives of cholestane-3,5,6-triol described by Pickard and Yates (15). They were prepared according to the methods of Rosenheim, Petrow, and Starling (16, 18, 19, 21). Westphalen (17) first made a diol derivative while attempting to acetylate the 5-OH group of cholestane-3,5,6-triol. Later, Rosenheim, Petrow, and Starling identified Westphalen's diol as 5-methyl-$\Delta^{8,9}$-norcholestene-3,6-diol and made it directly from cholesteryl acetate with heated solutions of selenium dioxide and identified a series of interconvertible isomers. All of these derivatives except 3,6-diacetoxy-5-methyl-$\Delta^{8,9}$-norcholestene and Δ^{5}-cholestene-3,4-dione, form B, exhibited the typical Liebermann-Burchard blue and violet to green color transformations on addition of the sulfonating reagent.

The 5-methyl-$\Delta^{8,9}$-norcholestene-3,6-diol was made by acetylation of cholestane-3,5,6-triol (Experiment 411) and treatment of the 3,6-diacetoxycholestane-5-ol in acetic anhydride with concentrated sulfuric acid at room temperature. The dehydration and isomerization were accompanied by a transient deep blue color typical of that produced by the Liebermann-Burchard test. Subsequently, upon sulfonating the colorless crystals of the norcholestenediol diacetate derivative, a change of color progressing from pink to red accompanied the reaction. Although this derivative responded negatively to the Liebermann-Burchard test, it became strongly antirachitic on sulfonation. Therefore, use of this color test as a means of identifying steroids which can be antirachitically sulfonated would appear to be valid only if applied to the mother substance. The diones cannot be transformed to antirachitic products on sulfonation in this manner (Experiment 507). Neither of the two unsulfonated derivatives (Experiments 478 and 508) produced healing in rachitic rats at the levels tested. Neither can cholestenone be anti-

TABLE III

Antirachitic Sulfonation of Oxidized Derivatives of Cholesterol

Experiment No.	Steroid material (and bibliographic reference No.)	Amount used	Reagents and amount used					Antirachitic activity as indicated by healing response of rachitic rats			
			Kind	Amount	AcOH	Ac_2O	Reaction temperature	No. of rats per group	Average steroid ⇔ fed per rat	Average gain or loss in weight	Average line test response,* plus values
		gm.		*ml.*	*ml.*	*ml.*	*°C.*		*mg.*	*gm.*	
411	Cholestane-3,5-, 6-triol (15)	0.2	H_2SO_4	0.06	4	0.2	117	3	5	2	1.3
								3	50	−5	4.4
477	3,6-Diacetoxy-5-methyl-$\Delta^{8,9}$-norcholestene (16) (17)	0.73	(HSO_3Cl	0.1	2.5)† +8	0.14	117	4	0.5	0	0.0
								4	5	1	2.5
								4	50	−16	4.0
478	"			0	0	0		3	30	4	0.0
484	*cis*-Δ^5-Cholestene-3,4-diol (18)	0.8	(HSO_3Cl	0.13	2.5) +12	0.17	117	8	5	1	1.6
								8	50	−7	4.8
								8	146	−15	5.0
495	"	1.6	("	0.5	2.5) +27	0.4	117	4	1	−2	0.0
								4	3	−1	1.1
								4	6	−3	2.3
								8	50	4	5.0
								8	146	−4	5.0
	"	1.6	("	0.5	2.5) +27	0.4	117	4	50‡	13	5.0
								4	146‡	0	5.0
505	"	1.6§	("	0.5	2.5) +27	0.4	117	4	5	6	1.7
								4	50	−4	5.0
								4	146	−8	5.0
511	"	0.8	("	0.26	2.5) +12	0.2	90	4	5	−1	2.0
								4	50	−5	4.0
								4	146	−14	5.0
512	"	1.6	("	0.26	2.5) +27	0.4	90	4	5	5	1.5
								4	50	−5	4.0
								4	146	−6	5.0
500	"	0.8	HSO_3Cl	0.4	15	0.2	117	4	5	0	1.8
								4	50	−6	3.9
								4	146	−9	5.0
499	Cholesterol	0.77‖	"	0.4	15	0.2	117	4	4.8	0	2.6
								4	48	−10	3.6
								4	140	¶	
496	3,6-Diacetoxy-Δ^4-cholestene (19)	0.96	(HSO_3Cl	0.13	2.5) +12	0.2	117	4	5	−1	1.9
								4	50	−17	3.7
								4	146	−22	5.0

TABLE III—*Concluded*

Experiment No.	Steroid material (and bibliographic reference No.)	Amount used	Reagents and amount used					Antirachitic activity as indicated by healing response of rachitic rats			
			Kind	Amount	AcOH	Ac_2O	Reaction temperature	No. of rats per group	Average steroid‡ fed per rat	Average gain or loss in weight	Average line test response,* plus values
		gm.		*ml.*	*ml.*	*ml.*	*°C.*		*mg.*	*gm.*	
497	3,4-Diacetoxy-Δ^5-cholestene (18)	0.96	(HSO_3Cl	0.13	2.5) +12	0.2	117	4	5	0	2.5
								4	50	−6	4.2
								4	146	−12	5.0
412	α-Δ^5-Cholestene oxide (20)	0.2	H_2SO_4	0.06	5	0.2	117	3	5	0	1.9
								3	50	−3	3.6
507	Δ^5-Cholestene-3,-4-dione, form B (21)	0.8	HSO_3Cl	0.26	15	0.2	117	4	5	5	0.0
								4	50	2	0.0
								4	145	2	1.6
508	"			0	0	0	0	4	25	13	0.0

* Negative control tests were conducted routinely for all experiments. No rats showed healing. Complete healing is indicated by 5.0 (6).

† The items included in the parentheses indicate that the reagents were combined as indicated prior to addition to the substrate in the acetic acid.

‡ Added to stock colony ration instead of rachitogenic ration, No. 2965 (6).

§ Duration of reaction, 5 hours in Experiment 505; in all others, 14 hours.

‖ Aerated during sulfonation.

¶ All rats died.

rachitically sulfonated ((1), p. 74), by this procedure. It is probable that oxidation to the ketone stage prevents antirachitic sulfonation of cholesterol derivatives.

The effect of varying the amount of sulfonating acid on the quantity of antirachitic activity produced from equal amounts of *cis*-Δ^5-cholestene-3,4-diol was studied. These results are reported in Table III (Experiments 484, 495, 500, and 505). They show that use of sulfonating acid in proportions ranging from 1 to 3 mole equivalents was without much effect in improving the resultant potencies.

In the case of the 3,4-diol derivatives, an increase in the sulfonating temperature above 90° for 14 hours (Experiments 505, 511, and 512) and time exceeding 5 hours at 117° (Experiments 495 and 505) appeared to produce similar antirachitic activity.

Eck and Thomas have reported (2) that the cholesterol derivative, cholestene-5, can be converted to an antirachitic product by the sulfuric acid-acetic anhydride sulfonating reagent. The oxidized derivative, α-Δ^5-cholestene oxide, produced by the oxidation of cholestene-5 with perbenzoic acid and subsequent dehydration (20), also yielded an antirachitic sulfonated residue having the usual potency (Experiment 412) following treatment with the same reagent as had been used in the earlier work to activate cholestene-5.

Antirachitic Activity and Growth Retardation—Examination of the changes in live weight of the rats during the test periods indicates that the amount of weight lost was correlated with the quantity of sulfonated product fed. Invariably the greatest losses in live weight occurred in those groups to which the largest amounts of sulfonated product were fed. These were at a maximum in the case of sulfonated 3,6-diacetoxy-5-methyl-$\Delta^{8,9}$-norcholestene (Experiment 477), 3,6-diacetoxy-Δ^4-cholestene (Experiment 497), and cholesterol (Experiment 499), and at a minimum in the sulfonated 3,4-diols. The relationship of isomerization in antirachitic sulfonation to both antirachitic potency and extent of growth retardation is suggested by the results of Experiments 477, 496, and 497. The 3,4 isomer was definitely less deleterious than the 3,6. The synthesis of the 3,6-diol differs mainly from that of the 3,4-diol in that the oxidation with selenium dioxide in acetic acid was carried out at 117° instead of 90°. This suggested that possibly in the analogous reaction with chlorosulfonic acid in antirachitic sulfonation a similar deleterious isomerization might occur. However, when the 3,4-diol was sulfonated at the lower temperature (Experiments 511 and 512), no less loss in weight was apparent.

SUMMARY

A study was made of various modifications of the procedure for antirachitic sulfonation. The modifications involved principally variations in the quantity of reagents used and time and temperature of the reaction. Relatively wide variations of conditions often led to insignificant changes in potency. Time and temperature were mutually compensating.

Without exception, hydroxysteroids, unlike a ketosteroid tested, could be antirachitically sulfonated. A Liebermann-Burchard positive steroid and its Liebermann-Burchard negative derivative were both antirachitically sulfonated.

The growth of rachitic rats fed rations fortified with chemosterol D as it is produced by present methods appears to be negatively correlated with the amount included in the ration.

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ON THE REQUIREMENT FOR DIPHOSPHOPYRIDINE NUCLEOTIDE IN THE AEROBIC METABOLISM OF PYRUVATE BY BRAIN TISSUE

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There appears to be general agreement that at least the following cofactors are necessary for the restoration of normal aerobic pyruvate utilization in brain suspensions after dialysis: (*a*) inorganic phosphate, (*b*) magnesium ions, (*c*) adenylic acid or adenosine triphosphate (ATP), and (*d*) a C_4-dicarboxylic acid. The participation of diphosphopyridine nucleotide (DPN) as an additional cofactor, however, has not as yet been unequivocally demonstrated. Thus Banga *et al.* (1), using dialyzed rabbit and pigeon brain dispersions, found that adenylic acid could be replaced by DPN under some conditions but not consistently. Green *et al.* (2), working with washed suspensions of kidney, liver, brain, and other organs, concluded that DPN was not a necessary cofactor in the oxidation of pyruvate by such preparations. Reiner (3), using a "complete system," containing all known cofactors and DPN as well, has presented manometric data on the utilization of pyruvate by rat brain homogenates which raise the question of whether or not pyruvate is utilized at all under these conditions.

In our own studies on the cofactors concerned with pyruvate metabolism in brain dispersions, we likewise obtained inconsistent results when the requirement for DPN was investigated. Further study, the results of which are presented here, indicates that DPN is in fact a necessary component in the aerobic metabolism of pyruvate by brain, and that past difficulties in demonstrating this fact have probably been due to lack of control of the inherent DPNase activity of brain tissue.

Methods

Adult Carworth Farms mice, without regard to sex, were used throughout this study. The animals were fasted for 18 hours prior to use and killed by crushing the neck with a forceps. The brain was excised down to but exclusive of the cerebellum, rinsed in distilled water, drained on filter paper, weighed on a torsion balance, and transferred to a glass homogenizer of the Potter-Elvehjem type (4) which had previously been chilled in an ice water jacket. To the brain material, 4 volumes of ice-cold distilled water, or other appropriate solutions as described below, were added and the whole was homogenized, with the homogenizer tube kept in the ice water jacket during this process. About 15 minutes ordinarily elapsed between brain removal and the end of homogenization.

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Aliquots (0.3 ml.) of the homogenates were transferred to the side arm of 17 ml. conical Warburg vessels previously charged in the main compartment with the following solutions: K pyruvate, 0.25 M, 0.6 ml.; DPN, 0.015 M, 0.2 ml.; ATP (as K salt), 0.0137 M, 0.2 ml.; cytochrome *c*, 0.0005 M, 0.35 ml.; K fumarate, 0.024 M, 0.2 ml.; nicotinamide, 0.60 M, 0.2 ml.; $MgCl_2$, 0.12 M, 0.2 ml.; KH_2PO_4-K_2HPO_4 buffer, pH 7.4, 0.40 M, 0.3 ml.; H_2O, 0.45 ml.

This is substantially identical with Reiner's "complete system" (3), in so far as cofactors are concerned, but with hexose diphosphate omitted. Each vessel contained 0.2 ml. of 20 per cent NaOH in the center well to absorb CO_2.

The vessels with attached manometers were transferred as quickly as possible to the constant temperature bath at 38°. This usually required about 10 minutes after homogenization. The vessels were shaken with air as the gas phase[1] for 10 minutes; a zero time reading was taken, the homogenates were added to the contents of the main vessel, and incubation was continued for a further 30 minute period. Readings of O_2 consumption were taken at 10 minute intervals. At the end of the incubation period, trichloroacetic acid to yield a final concentration of 5 per cent was added to each vessel. The vessel contents were removed quantitatively, adjusted to a known volume, and filtered through No. 42 Whatman filter paper. Aliquots of the filtrates were used for pyruvic acid analysis. In zero time controls, trichloroacetic acid was added to the vessel contents before the addition of the brain homogenate.

In the determination of dry weight, aliquot samples of each homogenate were removed immediately after preparation, dried at 105° for 24 hours, and weighed. When homogenates of different degrees of dispersion were prepared from the same tissue material, samples for dry weight determination were removed at every stage and at a time coinciding as closely as possible with the addition of such aliquots to the Warburg vessels.

Potassium pyruvate was prepared and recrystallized by an unpublished method.[2] The recrystallized material was found to be 98 per cent pure by alkali titration and by iodometric titration (7). *Adenosine triphosphate* was prepared from rabbit muscle as the barium salt by the method of LePage (8), and converted into a solution of its potassium salt. The concentration of ATP was established by determining the amount of inorganic phosphate formed on heating with N HCl in a boiling water bath for 15 minutes. Inorganic phosphate was determined by the method of Fiske and Subbarow (9).

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[1] In confirmation of the findings of Mann and Quastel (5) and Dickens (6) it has been found that both oxygen consumption and pyruvate disappearance are depressed in this system if oxygen is substituted for air in the gas phase.

[2] Kondritzer, A. A., personal communication.

Diphosphopyridine nucleotide was a commercial product (Schwarz Laboratories, Inc.), approximately 33 per cent pure; the degree of purity was determined by the increase in absorption at 340 mμ after reduction with dithionite according to LePage (10). To test for the presence of triphosphopyridine nucleotide (TPN) the TPN-linked malic decarboxylase system of Ochoa (11) was used.[3] No TPN activity could be detected when 6 mg. of our DPN preparation, equivalent to 2 mg. of DPN, were used. Since by the method in our hands a minimum of 19 γ of TPN could be detected, the TPN content of our preparation was less than 0.3 per cent. All solutions of DPN were adjusted to approximately pH 7.5 immediately before use.

Cytochrome c was prepared from beef heart by the method of Keilin and Hartree (12) and dialyzed against H_2O. The concentration of the solution so obtained was determined spectrophotometrically by the method of Drabkin (13). Fumaric acid was used in the form of the potassium salt.

Pyruvic acid was determined colorimetrically by reaction with 2,4-dinitrophenylhydrazine by a modification of the method described by Penrose and Quastel (14). It was not necessary to use the extraction procedure recommended by Friedemann and Haugen (15), since repeated control experiments showed consistently low values for the presence of substances other than pyruvic acid which reacted with dinitrophenylhydrazine; moreover, the concentration of such substances was the same at the beginning and end of the experimental period. DPN assays were carried out on heat-inactivated aliquots of experimental mixtures by the method of Jandorf *et al.* (16).

Results

In preliminary experiments, the oxygen consumption of mouse brain suspensions was studied with glucose (0.028 M) as substrate, and with the various cofactors described by Reiner (3), including hexose diphosphate (0.005 M). With this system Q_{O_2} values similar to those given by Reiner for rat brain were found. When glucose was replaced by 0.01 M pyruvate as substrate, an oxygen consumption of more than twice that found in the absence of any added substrate, as well as disappearance of pyruvic acid, was observed. The ratio (moles of oxygen consumed in the presence of pyruvate) to (moles of pyruvate disappearing) was approximately 1.1, in the same range though slightly lower than that found by Peters and his coworkers (1, 17, 18). When Q_{O_2} values were calculated from the period of linear oxygen consumption (usually the first 20 minutes of the experimental period), values of -15 to -25 were obtained.

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[3] We are indebted to Dr. S. Ochoa for a sample of purified TPN.

Our first experiments concerning the necessity for the various cofactors in the "complete system" demonstrated a consistent decrease in both oxygen consumption and pyruvate disappearance when cytochrome *c*, Mg^{++}, ATP, or fumarate was omitted (Table I). When DPN and nicotinamide were omitted, however, inconsistent results were obtained: little or no depresssion in Q_{O_2} and pyruvate disappearance in some cases, marked depression in others (compare Experiments 1 and 2, Table I).

TABLE I

Effect of Various Cofactors on Pyruvate Disappearance in Mouse Brain Homogenates

$-Q_{O_2}$ = microliters of oxygen consumed per hour per mg. of dry weight of tissue. System as described in the text with appropriate omissions.

Components of system	Experiment 1		Experiment 2	
	$-Q_{O_2}$	Pyruvate disappearance	$-Q_{O_2}$	Pyruvate disappearance
		γ per hr. per mg dry weight		*γ per hr. per mg. dry weight*
Complete	24	57	25	61
Less pyruvate	8		7	
" DPN and nicotinamide	16	49	10	26
" cytochrome *c*	8	39	11	52
" magnesium	6	20	11	33
" ATP	13	36	9	35
" fumarate	3	30	4	24

Investigation of these variations revealed that the need for the addition of DPN and nicotinamide could be correlated with the method of preparing the homogenates. Those prepared in a loosely fitting homogenizer tube showed little or no differences in O_2 consumption and pyruvate disappearance in the presence or absence of DPN and nicotinamide. When the tissue was homogenized for the same length of time and at the same rate of rotation of the pestle in a tight fitting tube,[4] it was invariably necessary to add DPN and nicotinamide to the system to restore its ability to metabolize pyruvate. No significant difference in temperature of homogenates prepared by either means, at the end of the period of homogenization, was discovered. Histological examination of stained smears of both coarse and fine homogenates revealed many clumps of intact nerve cells in the former, with an occasional free nucleus, and but few intact cells and free nuclei in the latter.

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[4] For the sake of brevity such homogenates will be referred to as "coarse" and "fine" homogenates, respectively.

In subsequent experiments it was found possible consistently to prepare coarse homogenates for which the presence or absence of added DPN had no effect on either oxygen consumption or pyruvate utilization, and fine homogenates which required added DPN. A series of such experiments is shown in Table II, where the effects of DPN and nicotinamide on coarse and fine homogenates are compared. The results demonstrate that when mouse brain is homogenized for 1 to 10 minutes in a loosely fitting homogenizer both the oxygen consumption and pyruvate disappearance are unaffected by omission of DPN and nicotinamide. Fine homogenates, on the other hand, show a marked depression in both these activities in the absence of added DPN and nicotinamide; normal activities are restored on addition of these substances. In the complete system the extent of homogenization affects neither oxygen consumption nor pyruvate disappearance. It may be noted that omission of Mg ions and cytochrome *c* appears to have the opposite effect; *i.e.*, a more marked depression of both metabolic activities in coarse than in fine homogenates. This phenomenon was not studied further.

TABLE II

Effect of Duration of Homogenization on Requirement of DPN and Nicotinamide Addition to Coarse and Fine Brain Homogenates

Preparation of homogenates and components of system as described in the text.

Coarse homogenate					Fine homogenate				
Complete system			Less DPN and nicotinamide		Complete system			Less DPN and nicotinamide	
	$-Q_{O_2}$	Pyruvate disappearance	$-Q_{O_2}$	Pyruvate disappearance		$-Q_{O_2}$	Pyruvate disappearance	$-Q_{O_2}$	Pyruvate disappearance
min.		*γ per hr. per mg. dry weight*		*γ per hr. per mg. dry weight*	*min.*		*γ per hr. per mg. dry weight*		*γ per hr. per mg. dry weight*
1	21	51	20	46	1	21	50	11	33
1	22	56	20	50	6	24	96	13	27
6	22	66	21	56	6	18	55	13	29
10	21	52	20	42	10	24	54	12	25

These results with coarse and fine homogenates may be explained by assuming that thorough disintegration of brain tissue releases the intracellular enzyme, DPNase, or activates it in some way, and thus promotes the decomposition of endogenous DPN; in less thoroughly ground material, on the other hand, DPN is somehow unavailable to the cell DPNase, and remains intact to such an extent as to sustain normal respiration and pyruvate catabolism.

Work by Mann and Quastel (19), Handler and Klein (20), and others has shown that DPNase is inhibited in the presence of concentrations of nicotinamide of the order of 0.01 M. This finding has already been utilized in the preceding experiments, in which nicotinamide had been added to fine homogenates together with DPN to prevent further breakdown of the added DPN. If our hypothesis concerning the differences between coarse and fine homogenates were correct, fine homogenates prepared in the presence of sufficient nicotinamide should show normal pyruvate metabolism unaffected by further addition of DPN. Table III presents evidence to substantiate this prediction. In these experiments various samples of brain tissue were homogenized for 6 minutes in a tightly fitting homogenizer with 4 volumes of an ice-cold solution of nicotinamide instead of water. It may be seen from Table III that a concentration of 0.5 per cent nicotinamide during homogenization is not sufficient to obviate the necessity of adding further DPN for full restoration of pyruvate metabolism. Concentrations above 1 per cent of nicotinamide, however, yield homogenates with normal oxygen consumption and pyruvate utilization in the presence or absence of added DPN. When the nicotinamide concentration was raised to 4 per cent or above, an inhibition of oxygen consump-

TABLE III

Effect of Homogenization in Nicotinamide Solutions on Necessity of DPN Addition to Fine Brain Homogenates

All homogenates were prepared by grinding 1 part of brain with 4 parts of nicotinamide solution for 6 minutes in a tightly fitting homogenizer.

	Complete system		DPN and nicotinamide omitted	
Nicotinamide concentration*	$-Q_{O_2}$	Pyruvate disappearance	$-Q_{O_2}$	Pyruvate disappearance
gm. per 100 ml. solution		*γ per hr. per mg. dry weight*		*γ per hr. per mg. dry weight*
0.5	21	45	10	12
0.7	20	49	12	32
1.0	26	46	21	44
1.4	24	52	18	44
2.7	24	47	24	42
4.1	15	52	15	51

* The concentrations of nicotinamide are those of the solutions used for homogenization; the *final* concentration of nicotinamide after addition of homogenate to the rest of the system is one-tenth of the above concentrations.

tion with or without added DPN was found, while pyruvate utilization was unimpaired and independent of the addition of DPN. The nature of this inhibition, which has previously been noted by Mann and Quastel (19), has so far not been elucidated; it has been noted, however, that it cannot be reversed by the addition of excess DPN.

The above experiments provide indirect support for the theory that fine homogenates have a higher DPNase activity than coarse homogenates. More direct evidence is provided by following the rate of decomposition of endogenous DPN in coarse and fine homogenates, as shown in Table IV. In these experiments, coarse and fine homogenates were incubated for various lengths of time at 38°, then heated in a boiling water bath for 15 minutes, and centrifuged. Each supernatant was decanted, the precipitate washed with 0.5 to 1 ml. of water, recentrifuged, and the washings and supernatant combined. This solution was then used for the direct determination of DPN by the method of Jandorf *et al.* (16). Control experiments demonstrated maximal and uniform extraction of DPN by this means. It is apparent from the results given in Table IV that the DPN content of a fine homogenate decreases at a much greater rate upon incubation at 38° than is the case with a coarse homogenate prepared from the same tissue material. This is further evidence of an increased DPNase activity in fine homogenates over that present in coarse homogenates. Furthermore, it has been consistently noted that in fine homogenates the decrease in DPN content on incubation is roughly parallel to the decrease in oxygen consumption.

TABLE IV

DPN Content of Coarse and Fine Mouse Brain Homogenates after Incubation at 38° for Various Lengths of Time

Length of incubation	DPN content of homogenate	
	Coarse	Fine
min.	*γ per gm. dry weight*	*γ per gm. dry weight*
0	670	620
10	531	370
20	472	250

DISCUSSION

The experiments presented in this paper demonstrate that pyruvate is metabolized in our mouse brain preparations, as evidenced by disappearance of this substrate from the system. No attempt has been made to determine the fate of the pyruvate thus metabolized; it seems unlikely that all of it is oxidized to CO_2 and H_2O, since such metabolism would require a value of 2.5 for the molar ratio of O_2 consumed to pyruvate metabolized, in contrast to our observed ratio of approximately 1.1; this is in agreement with Long's (18) findings that in pigeon brain about two-thirds of the metabolized pyruvate is completely burned, the remainder being accounted for as acetate and lactate.

In addition, these experiments demonstrate clearly that evidence as to substrate utilization must be based primarily on direct chemical determination rather than on the indirect evidence of oxygen consumption. Thus, high concentrations of nicotinamide were found to depress appreciably the uptake of oxygen by our system without any marked effect on the pyruvate disappearance.

The involvement of DPN in the metabolism of pyruvic acid here studied has been demonstrated by various means. First, in suitable mouse brain preparations, it has consistently been possible to show that in the absence of added DPN both oxygen consumption and pyruvate utilization are significantly impaired. Secondly, both of these metabolic activites can be restored to original levels by the addition of an active DPN preparation. Lastly, both oxygen consumption and pyruvate disappearance can be maintained at normal levels by preparing brain homogenates in the presence of nicotinamide in concentrations which have been shown to prevent the breakdown of DPN (19).

The question arises as to whether the restorative activity of our DPN preparations is indeed due to DPN itself rather than to an accompanying factor. The substances likely to be present in such a DPN concentrate, prepared by the method of Williamson and Green (21), are adenylic acid and its phosphorylated derivatives, and TPN. The first group of substances can be excluded, since concentrations of ATP, more than 3 times the equivalent of the amount of DPN necessary to restore full activity, were completely inactive. TPN may be excluded since (*a*) it comprised at most 0.3 per cent of the DPN preparation used (a value within the limit of detection of the malic decarboxylase test system), and (*b*) an amount of purified TPN, equivalent to an amount of DPN necessary to restore almost complete activity to a brain homogenate, was likewise inactive in this respect.

The results reported here for mouse brain are in contrast to the findings of Green *et al.* (2) pertaining to pyruvate metabolism by the washed insoluble particles of various mammalian tissues. According to these authors pyruvate is oxidized completely without the addition of any pyridine nucleotides, the only cofactors necessary being Mg^{++}, adenylic acid, and a C_4-dicarboxylic acid. We are unable to reconcile these divergent results; they may be due to differences in the methods of tissue preparation.

Indeed this study shows that seemingly small variations in the technique of preparing homogenates may have a profound effect on the metabolic requirements and activities of the resulting preparations. Such differences between coarsely and finely ground tissue dispersions as have been reported by Stadie *et al.* (22) for the effect of insulin on pyruvate metabolism in muscle, by Gibson and Long (23) for pyruvate metabolism in the heart, and by Novikoff *et al.* (24) for the DPN content of embryos may well have been due to a variable release or activation of DPNase and consequently variable DPN destruction.

Barron *et al.* (25) have recently reported that ground lung tissue exhibits a markedly lower metabolism of pyruvate and other substrates than do lung slices. These workers also attribute their findings to the high DPNase activity of lung tissue. Their results are in good agreement with those reported here concerning brain tissue.

It is unfortunate that no more precise criteria for the state of dispersion of a given tissue are available than the qualitative classification as "coarse" and "fine" homogenates used in this study as well as by other workers in the field. Schneider and Potter (26) have attempted to establish a "cytolysis quotient" as a measure of cell rupture; later work, however, has shown this quotient to be a measure of some subcellular entity rather than of the state of cell intactness (27). Measurement of DPNase activity may provide a more reliable measure of cellular destruction.

The authors are indebted to Dr. S. H. Durlacher, Pathology Section, for histological study of some of the brain homogenates here employed.

SUMMARY

1. Diphosphopyridine nucleotide (DPN) is an essential cofactor in the oxidation of pyruvic acid by water-homogenized mouse brain preparations. Other cofactors required for the demonstration of aerobic pyruvate metabolism with such preparations include inorganic phosphate, Mg^{++}, adenosine triphosphate, fumarate, and cytochrome *c*.

2. The requirement for the addition of DPN to homogenized brain preparations to promote pyruvate oxidation becomes apparent only when the tissue is highly homogenized. Coarse homogenates do not require added DPN in pyruvate oxidation. It is not necessary to add DPN to highly homogenized preparations if homogenization is conducted in 1 to 3 per cent nicotinamide solutions instead of in water alone.

3. Evidence is presented that the liberation or activation of the intracellular enzyme DPNase is of importance in the demonstration of the rôle of DPN in pyruvate oxidation by brain homogenates. Highly homo-

genized brain preparations have a more marked DPNase activity than coarse homogenates and nicotinamide presumably inhibits such enhanced activity. Conflicting results by previous workers on the requirement for DPN in pyruvate metabolism by brain tissue may have been due to variations in the mode of tissue preparation.

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THE BIOLOGICAL ACTIVITY OF SUBSTITUTED PYRIMIDINES*

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Recent investigations have shown that the animal microorganism, *Tetrahymena geleii*, requires an exogenous source of pyrimidine for growth (1). This requirement can be met by the addition of cytidylic acid or uracil to the medium (2). Cytosine and thymine are without activity. The failure to utilize the latter two free bases was interpreted as indicating a block in the ability of the organism to carry out the riboside linkage in these two cases. The utilization of uracil, however, demonstrates the ability of the organism to carry out the specific riboside linkage to form the nucleoside and nucleotide of uracil and also its ability (and obligation) to substitute an amino group for the keto group in the 6 position without breaking the riboside linkage. Of the naturally occurring pyrimidine bases, therefore, uracil was found to be the only one which would satisfy the needs of this organism.

A systematic testing of various substituted pyrimidine bases over wide ranges of concentrations offers a means of evaluating the physiological importance of the positions and radicals. This, in turn, may help us to understand better the particular enzymes involved. The substituted pyrimidines can be divided into four categories: (1) they may replace uracil; (2) they may exert sparing action, so that the amount of uracil required for optimal growth is reduced; (3) they may exhibit inhibition even in the presence of uracil; and (4) they may be inert.

The purpose of this communication is to report the results obtained on twenty-three substituted pyrimidine bases in regard to their activities when tested with *Tetrahymena*. We are deeply grateful to Dr. George H. Hitchings and his colleagues at the Wellcome Research Laboratories for generously supplying us the pyrimidines used. Without their cooperation this study would have been impossible.

Material and Methods

The organism used in these investigations was *Tetrahymena geleii*, strain W, grown in pure (bacteria-free) cultures. The basal medium is given in Table I. Growth was measured turbidimetrically (5) and all experiments

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were repeated a varying number of times. Within any single experimen triplicate tubes were invariably used and the results expressed as averages

When tests were set up for replacement or sparing action, the result were all based on third serial transplants in identical concentrations of components. In all experiments involving inhibitory compounds, however it was found that completely reproducible results could be obtained only when the organisms used for inoculation into medium containing an inhibitor were grown for two serial transplants in a medium containing suboptimal level of the antagonist (*e.g.*, uracil). This technique is necessary to avoid permanent injuries (in the case of some of the more poten inhibitors) and any tendency to adaptation to the inhibitor. Incubatio was for 72 hours (unless otherwise noted) at 25°.

Each compound was tested for replacement over a range of graded concentrations up to 100 γ per ml. of medium, in the absence of other py

TABLE I

Basal Medium

All amounts are given in micrograms per ml. of final medium.

L-Arginine HCl	83	Nicotinamide	0.10
L-Histidine HCl	36	Thiamine HCl	1.00
DL-Isoleucine	113	Riboflavin	0.10
L-Leucine	147	Pteroylglutamic acid	0.01
L-Lysine HCl	116	Pyridoxine HCl	0.1
DL-Methionine	94	Pyridoxal HCl	0.01
L-Phenylalanine	70	Pyridoxamine HCl	0.01
DL-Threonine	138	Biotin (free acid)	0.0005
L-Tryptophan	28	Choline Cl	1.00
DL-Valine	76	Protogen[*]	0.375
DL-Serine	157	Guanylic acid	30
L-Glutamic acid	233	Adenine	20
L-Aspartic acid	61	$MgSO_4 \cdot 7H_2O$	100
Glycine	5	K_2HPO_4	100
DL-Alanine	55	$CaCl_2 \cdot 2H_2O$	50
L-Proline	175	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25
L-Hydroxyproline	75	$FeCl_3 \cdot 6H_2O$	1.25
L-Tyrosine	67	$CuCl_2 \cdot 2H_2O$	5
L-Cysteine	3.5	$MnCl_2 \cdot 4H_2O$	0.05
Dextrose	2500	$ZnCl_2$	0.05
Sodium acetate	1000	Tween 85[†]	700
Ca pantothenate	0.10		

[*] Furnished by the Lederle Research Laboratories through the courtesy of Dr E. L. R. Stokstad. Protogen represents a portion of the previously used "Facto II." For the references see (3) and (4).

[†] Furnished by the Atlas Powder Company, Wilmington, Delaware.

rimidine. A similar range of concentrations was then tested with suboptimal amounts of uracil (2 γ per ml.) or cytidylic acid (5 γ per ml.) to determine whether or not any sparing action could be demonstrated. Possible inhibitory action was tested for in graded concentrations up to 500 γ per ml., in the presence of at least two suboptimal levels of uracil. The low solubility of many of the compounds precluded satisfactory use of higher concentrations.

TABLE II

Replacement	Per cent activity*	Inhibition	Inhibition index†	Sparing	Inert
1-Methyluracil	1.00	2-Thio-4-aminopyrimidine	<20	4-Methyluracil	2-Amino-4-hydroxypyrimidine (isocytosine)
3-Methyluracil	0.84	5-Hydroxyuracil (isobarbituric acid)	25		1,3-Dimethyluracil
5-Bromouracil	0.50	2-Thio-6-ketopyrimidine (2-thiouracil)	100		5-Methylcytosine
5-Nitrouracil	0.12	2,4-Diaminopyrimidine	100		4-Ketouracil (barbituric acid)
		5-Aminouracil	200		4-Hydroxypyrimidine
		2,4-Diamino-6-methylpyrimidine	300		4,5-Dihydroxypyrimidine
		2,4-Diamino-5-formylamino-6-hydroxypyrimidine	300		5-Methyl-6-hydroxypyrimidine
		2-Aminopyrimidine	>1000		Cytosine
					Thymine

* Calculated from uracil, which was taken as 100 per cent.

† The inhibition index may be defined as the smallest ratio between the amount of the inhibitor and antagonist at which growth does not occur.

Results

Table II summarizes the results obtained. Of the substituted uracils tested, four were capable of supplying the pyrimidine requirement. Their activities were very low, however, when compared with uracil. Methylation of either the 1 or 3 position reduced the activity to 1 per cent or less. These percentages were calculated by comparing the amounts of uracil (rated as 100 per cent) with that of the substituted uracil required to produce equal growth. Substitution in position 5 with either —Br or —NO_2 reduced the activity to 0.5 and 0.12 per cent respectively. These latter activities are so low as to suggest the possibility of slight uracil contamination.

Sparing action, but not replacement, results when position 4 i methylated (4-methyluracil). Maximum effect is not shown, however until after long periods of incubation (96 hours and over). There is n indication from our data as to the active pyrimidine formed and it appear that, while positions 1, 3, and 5 may be hydrogenated to form uracil, position 4 can be hydrogenated only in the presence of some uracil. It mus be remembered, however, that 5-methyluracil (thymine) is inactive fo replacement (2) and shows no sparing action, so the hydrogenation of th 5 position is more difficult when —CH_3 is present than when either —B or —NO_2 is present.

When both positions 1 and 3 of uracil carry —CH_3 groups (1,3-dimethyluracil), then all activity is destroyed. The difficulties of hydrogenatin these positions singly appear to be additive to such an extent that th enzyme systems are incapable of converting this compound to an activ form. Similarly, when the substitution is a keto radical in position 4 (barbituric acid), all activity is lost (no sparing action, as contrasted wit 4-methyluracil).

Eight of the compounds tested were found to be competitively inhibitory, inhibition being antagonized by uracil or cytidylic acid. They range in inhibitory activity from an inhibition ratio of 20 to over 1000. In al cases inhibitory activity depended upon the degree of resemblance of th compound to uracil. Those which bore the closest resemblance, havin substitutions at positions 2 and 4 or 2 and 6, are the most inhibitory. I is to be noted that positions 4 and 6 are very nearly equivalent, except fo the double bonds. Additional substitutions at position 5 modify th inhibitory effect.

In the case of the inert compounds, the explanation probably is tha enzyme systems are lacking in *Tetrahymena*, which would enable it to convert them to active forms. The lack of inhibition may be due to configurations of these compounds such that they fail to fit existing enzym surfaces. Cytosine is inert, as was discussed in a previous paper (2) because of the apparent failure on the part of the organism to perform th riboside linkage.

The results shown in Table II and discussed above may prove useful in manner not immediately apparent. Inasmuch as *Tetrahymena* is a organism with a rather typical animal biochemical pattern (6), it shoul prove useful in screening possible bacterial inhibitors. Thus the dat presented in Table II indicate that, if any compound in the categories othe than that of inhibition exhibit marked bacteriostatic action, then thos compounds may prove to be useful therapeutic agents. Hitchings *et al* (7) have recently shown that 5-nitrouracil is inhibitory to *Lactobacillu casei*. This compound shows no inhibitory action to *Tetrahymena*.

SUMMARY

Twenty-three substituted pyrimidine bases were tested for replacement, sparing action, and inhibitory action, by using the animal microorganism *Tetrahymena geleii.* Changes from the active pyrimidine base uracil result in the following: *position* 1, reduces activity; *position* 2, destroys activity when unsubstituted, and inhibition results when substituted with either $=S$ or $-NH_2$ (one exception); *position* 3, reduces activity; *position* 4, reduces activity to sparing only ($-CH_3$) or destroys activity ($=O$); *position* 5, reduces activity ($-NO_2$, $-Br$), destroys activity ($-CH_3$), or produces inhibition ($-NH_2$); *position* 6, destroys all activity.

It is suggested that these and similar results might be employed in tests for useful bacteriostatic compounds.

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ON THE UTILIZATION OF EXOGENOUS SULFATE SULFUR BY THE RAT IN THE FORMATION OF ETHEREAL SULFATES AS INDICATED BY THE USE OF SODIUM SULFATE LABELED WITH RADIOACTIVE SULFUR

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There has been much controversy regarding the origin of the sulfate sulfur for the detoxication of phenols. Baumann (1), on the basis of rather meager data obtained on two dogs, was of the opinion that exogenous sulfate could augment the excretion of phenol as phenyl sulfate. Tauber (2) and Rhode (3), however, using dogs and rabbits, respectively, were unable to confirm Baumann's observations. Hele (4), on the other hand, was successful in demonstrating that sodium sulfate as well as sodium bisulfite and cystine when administered by mouth with guaiacol carbonate increased the ethereal sulfate sulfur excretion of dogs. Subsequently, Hele (5) also showed that the dog can utilize sodium sulfate, given orally or subcutaneously, to form phenyl sulfate and indoxyl sulfate, and he suggested "that in every case ethereal sulphate, whether arising endogenously or exogenously, is formed by the union of the sulphate ion with phenolic compounds."

A more detailed summary of the controversial evidence has been given by Hele (4) and, more recently, by Williams (6).

One of the sites of ethereal sulfate formation was indicated by Embden and Glaessner (7) to be the liver. Marenzi (8) subsequently demonstrated that a liverless dog was able to conjugate phenol and that the small intestine possesses this ability to a remarkable extent. In this respect, Arnoldt and De Meio (9) have more recently shown that *in vitro* the intestine is more active than the liver in the synthesis of a phenol conjugate.

The synthesis of ethereal sulfates is indicated by the work of Bernheim and Bernheim (10) to be under the influence of labile enzyme systems. Their experiments with guinea pig liver slices give, in addition, support for the view that sulfate is used in the synthesis of ethereal sulfates, since they found that the conjugation of phenol was abolished in the absence of sulfate.

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On the basis of the above it seems likely that exogenous sulfate sulfur i used, at least in part, in the synthesis of ethereal sulfates and that thi synthesis takes place largely in the small intestine and liver. It was o some interest to see if this opinion could be further substantiated by th use of sodium sulfate, labeled with radioactive sulfur (S^{35}).

EXPERIMENTAL

Adult, male rats, 200 to 250 gm. in weight, from the colony of Professo E. V. McCollum, were given sodium sulfate,[1] containing S^{35}, by stomac tube or by intraperitoneal injection. In some instances phenol in aqueou solution was given by stomach tube at the same time. The animals wer housed in special metabolism cages suspended over large glass funnel To facilitate collection of urine specimens with a minimal contaminatio by the feces, the funnels were loosely stoppered with hobnailed glass ball

The urinary inorganic sulfate sulfur, total sulfate sulfur (11), and th total sulfur (12) were isolated as barium sulfate. The fecal sulfur wa likewise isolated as barium sulfate after oxidation according to the directions of Bailey (13). All barium sulfate samples were prepared for th determination of radioactivity, and their activity determined as previously described (14, 15).

As a check on the completeness of collection the creatinine content some of the urine specimens was determined by an adaptation of Folin method (16) for use with a photoelectric colorimeter.

RESULTS AND DISCUSSION

In order to determine the amount of S^{35} excreted in the urine and fece after intraperitoneal or oral administration of relatively large amounts labeled sodium sulfate, 50 mg. of sodium sulfate, containing S^{35}, were give to one rat by stomach tube and to a second by intraperitoneal injectio These rats were allowed food and water at all times during the course the experiment. The excretion of the radioactive sulfur in the urine a feces was followed for a period of 3 days. The data are summarized Table I. An examination of Table I indicates that a major portion of th S^{35} activity is excreted in the urine and feces in the first 24 hours. By th end of 72 hours it was found that elimination of the S^{35} by these routes excretion accounted for 85.6 per cent after oral administration and 79 per cent after intraperitoneal injection of the activity administere

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[1] The sodium sulfate, containing the S^{35}, was recovered according to the dire tions of Tarver and Schmidt (18) from the urines of rats given S^{35} as sodium sulfi The author is sincerely grateful to Dr. M. D. Kamen, Washington University, S Louis, Missouri, for a gift of irradiated carbon tetrachloride from which the label sodium sulfide was prepared.

These figures approach those previously reported for the excretion of S^{35} after the intraperitoneal injection of only 1 mg. of labeled sodium sulfate (15). On the basis of these figures it was decided to proceed as follows in an attempt to demonstrate the possible use of exogenous sulfate in the synthesis of ethereal sulfates.

Each of four rats was given 25 mg. of sodium sulfate, containing S^{35}, by stomach tube and then placed in a separate metabolism cage. No food was allowed. The urine collected in the following 24 hours and the cage washings were combined. The activity of the S^{35} in the inorganic sulfate, total sulfate, and total sulfur fractions of each rat's urine was determined. For the next 5 days the rats were allowed both food and water. The urine samples were not collected. On the 7th day the rats were again placed in clean metabolism cages without food and the urine collected for the following 24 hours. The urinary sulfur was again fractioned. A similar procedure was followed after the rats received 25 mg. of the labeled sodium sulfate by intraperitoneal injection. The observations on these urine specimens are summarized for one rat in Table II. Table II shows also the effect of the simultaneous administration of 25 mg. of phenol and labeled sodium sulfate on the activity of the S^{35} appearing in the ethereal sulfate sulfur fraction of the urine.

TABLE I

Excretion of S^{35} in Urine and Feces of Adult Rats after Oral and Intraperitoneal Administration of Labeled Sodium Sulfate

Each rat received 50 mg. of sodium sulfate, containing S^{35} (1900 counts per minute). Rat S-0 received the sodium sulfate by stomach tube, Rat S-1 by intraperitoneal injection.

Day	Total sulfur	Rat S-0		Rat S-1	
		Counts per min. in 24 hr. specimen	Counts recovered	Counts per min. in 24 hr. specimen	Counts recovered
			per cent		*per cent*
1	Urinary	1310	69.0	1426	75.0
	Fecal	191	10.0	23	1.2
2	Urinary	44	2.3	26	1.4
	Fecal	23	1.2	20	1.0
3	Urinary	46	2.4	8	0.4
	Fecal	13	0.7	5	0.2
Total 3 day excretion....		1627	85.6	1508	79.2

From the data in Table II it seems logical to conclude that exogenous sulfate sulfur is utilized by the rat in the synthesis of ethereal sulfates. This conclusion is consistent with the observations of Baumann (1) and of

Hele (4, 5) on the dog and of Bernheim and Bernheim (10) on guinea pig liver slices. It is in agreement with the conclusion reached by Laidlow and Young (17) in a note which appeared after this work was completed. The latter authors also used rats, to which sodium sulfate labeled with S^{35} and 2-naphthylamine were administered. The ethereal sulfate of 2-amino-1-naphthol was isolated and shown to contain radioactive sulfur.

Table II

Urinary Excretion of S^{35} by Adult Rat in 24 Hours after Oral and Intraperitonea Administration of Labeled Sodium Sulfate with and without Phenol

25 mg. of sodium sulfate containing S^{35} (2250 counts per minute) and 25 mg. o phenol were administered as indicated. The time interval between experiments i at least 6 days. The radioactivity measurements are corrected for decay and self absorption.

Experiment No.	Chemical analyses					Radioactive measurements				Remarks
	Creatinine	Inorganic sulfate S	Total sulfate S	Total S	Ethereal sulfate S	Inorganic sulfate S	Total sulfate S	Total S	Ethereal sulfate S	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*counts per min.*	*counts per min.*	*counts per min.*	*counts per min.*	
A	4.4	9.4	10.0	12.9	0.6	781	830	838	49	Na_2SO_4, orally
B	5.2	4.4	4.9	9.0	0.5	47	47	50	0	7th day after Experiment A
C	4.4	8.9	9.6	12.4	0.7	1150	1235	1220	85	Na_2SO_4 intraperitoneally
D	4.5	5.4	6.0	9.2	0.6	48	48	50	0	7th day after Experiment C
E	4.4	6.0	10.3	12.2	4.3	946	1178	1196	232	Na_2SO_4 and phen orally
F	5.5	9.5	14.2	21.5	4.7	806	1227	1245	421	Na_2SO_4 intraperitoneally; phen orally
G	4.2	4.5	7.6	9.9	3.1	635	936	940	301	Na_2SO_4 intraperitoneally in 4 doses, hr. apart; phen orally at beginnin

It was not possible to demonstrate, under the conditions of these exper ments, that the synthesis of phenyl sulfate after oral administration c sodium sulfate and phenol was greater than the synthesis when sodiur sulfate was given by intraperitoneal injection and the phenol orally.

If one defines specific activity as counts per minute per mg. of S, it wi be found on calculation from the data in Table II that the specific activit of the inorganic sulfate sulfur is in close agreement with the specific activ ities of the total sulfate sulfur and ethereal sulfate sulfur fractions in number of the experiments. For example, in the case of Experiment A, Table II, the specific activity of the inorganic sulfate sulfur fraction is 82, that of the total sulfate sulfur and ethereal sulfate sulfur fractions 83 and 80, respectively. An agreement similar to or approaching the one just cited was found in twelve out of the twenty-eight experiments done on the four rats. Such an agreement in the specific activities suggests that it is sulfate as such which is directly utilized in the *in vivo* synthesis of ethereal sulfates. This agreement in the specific activities may be an indication that, if sulfur from other sources than preexisting sulfate is used in the *in vivo* synthesis of ethereal sulfates, the sulfur is first converted to sulfate sulfur before it is conjugated with phenols. Hele (5) has made this suggestion and the work of Bernheim and Bernheim is also indicative of this possibility.

SUMMARY

Rats given sodium sulfate containing S^{35} incorporate some of the sulfur into the ethereal sulfate sulfur fraction of the urine. The amount incorporated can be increased by the simultaneous administration of phenol.

On the basis of the close agreement in the specific activities of the inorganic sulfate, total sulfate, and ethereal sulfate sulfur fractions in a number of the experiments it is suggested that, if sulfur from sources other than preexisting sulfate is used in the *in vivo* synthesis of ethereal sulfates, the sulfur is first converted to sulfate before it is conjugated with phenols.

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PARTITION CHROMATOGRAPHIC SEPARATION OF ADENINE AND GUANINE

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In the course of an investigation into the metabolic pathways of nucleotide purines by means of tracer elements, it became necessary to prepare from biological sources pure specimens of adenine and guanine in sufficient quantities for analyses in the mass spectrometer. In general, for preparative purposes, use has been made of the insolubility of adenine and guanine under certain strictly specified conditions. For small quantities of purines, however, these methods are unsatisfactory because of the difficulties in maintaining the specified conditions in small volumes. The employment of the liquid-liquid distribution principle in the form of partition chromatography seemed more promising as a separatory procedure for small amounts of purine, especially since Vischer and Chargaff (1) have shown that it is possible to separate adenine and guanine on paper strip chromatograms in the system quinoline-collidine-water. After the conclusion of our work a paper by Tinker and Brown (2) was brought to our attention. They employed the system butanol-1 M phosphate buffer, pH 6.5, for the separation of the purines in the Craig apparatus.

In our work partition chromatography on starch (3, 4) has effected the desired separation of adenine and guanine.

Results

For two reasons our choice of solvents has been restricted. In the first place we prefer not to have foreign nitrogen in the system. Secondly, it was desirable to follow the development of the chromatogram directly in the effluent. This could be conveniently done by taking advantage of the fact that the purines absorb strongly in the ultraviolet region and therefore the absorption of the solvent should be negligible. In preliminary experiments, we first chose *n*-butanol as a solvent, but the purines proved to be insufficiently soluble in this medium. The addition, however, of a small amount of the monomethyl ether of ethylene glycol to the butanol increased the solubility of the purines, and this solvent mixture was found to serve as an excellent developing agent in the chromatography.

In our first experiments the purines were put on the column as hydrochlorides. At that time the separation was never complete, because the zones displayed a tendency to spread and, furthermore, it was difficult to obtain quite reproducible results. Later sodium hydroxide was substituted for hydrochloric acid and this resulted in a considerable sharpening of the zones and an excellent reproducibility.

EXPERIMENTAL

The starch was prepared by extraction with methanol for 24 hours in a Soxhlet apparatus and drying *in vacuo* at 45°. The starch was then exposed to air and used directly in the columns. For the preparation of the column and the collection of the effluent a paper by Edman (5) should be consulted.

Solvents—The *n*-butanol was freed from impurities giving light absorption in the ultraviolet. This was done by refluxing the butanol with zinc dust and strong alkali for 2 hours and then distilling and redistilling *in vacuo*. Butanol (865 ml.) and water (135 ml.) were mixed.

The methylene glycol (practical grade) was distilled twice *in vacuo* before use.

To the 14 volumes of the butanol-water mixture was added 1 volume o methylene glycol. This solvent mixture was then used in the chromatography.

Estimation of Adenine and Guanine in Effluent—This was done by measuring the ultraviolet absorption of the effluent in a Beckman spectrophotometer in 1 cm. quartz cells at wave-lengths 262 and 248 mμ. The pure solvent served as a blank. The value of the quotient E_{262}/E_{248} differs widely for the two purines, being in the solvent mixture 1.50 for adenine and 0.7 for guanine. This quotient offers a convenient way of identifying the purines. It should be mentioned that the values of these quotients varied somewhat from one run to another, owing mainly to a small variable amount of absorbing material released from the starch. For the purpose however, of following the development of the chromatogram minor deviations from the correct quotients can be neglected. Whenever a greater accuracy was called for, the organic solvent was evaporated and the absorption of the purines measured in 1 N HCl.

Procedure—The extraction of the nucleotides from the cells and their fractionation into polydesose and polyribose nucleotides were carried ou according to Hammarsten (6). The fractions of polyribose and of polydesose nucleotides were hydrolyzed with sulfuric acid.

The purines were precipitated as their silver compounds according to th procedure described by Kerr and Seraidarian (7). The silver compound were carefully washed until the supernatant gave no reaction to sulfat ions. Silver nitrate was added during the washing procedure. The las traces of sulfate ions, which adhere very firmly to the silver precipitat are removed by the further addition of 0.1 ml. of a 1 per cent solution barium nitrate to the silver nitrate solution used for washing. Excess barium is removed by continued washing with the silver nitrate solution. In order to remove silver nitrate the precipitate is finally washed once with absolute ethanol and twice with ether.

Preparation of Solution of Mixed Purines—The silver compounds are decomposed with hydrochloric acid as described by Kerr and Seraidarian (7) and the hydrochloric acid removed through evaporation to dryness *in vacuo* three times. The purines are taken up in 2 to 3 ml. of distilled water, transferred to a wide test-tube, and the solution evaporated to dryness on the boiling water bath in a stream of air. It is important that the purines be deposited on the smallest possible area, because otherwise it is difficult to dissolve all the guanine. To the dry material are added 0.10 ml. of 1 N NaOH and 0.70 ml. of methylene glycol. The suspension is then heated very quickly over a free flame and 9.8 ml. of *n*-butanol-water mixture (87.2 ml. + 12.8 ml.) are added. The solution (10.6 ml.) is introduced into the starch column (35 mm. in diameter and 120 mm. long) and a chromatogram is developed. This procedure was used for the fractionation of guanine and adenine in a study of the nitrogen turnover in cell nuclei and cytoplasm in rat liver (8).

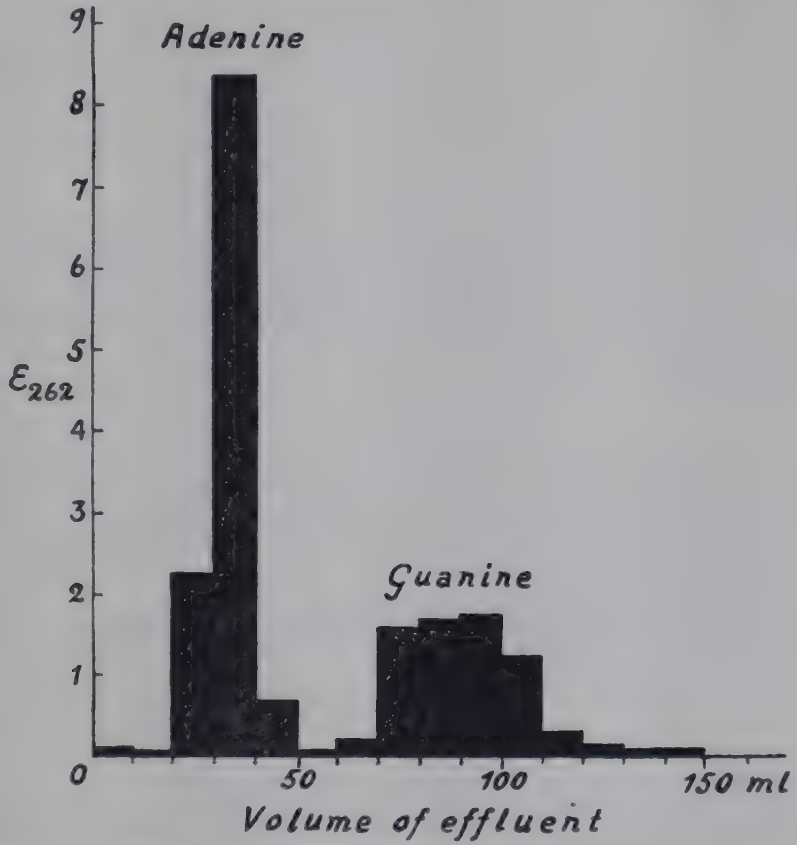

FIG. 1. Separation of adenine and guanine

Model Experiment on Pure Guanine and Adenine—In Fig. 1 the course of a typical experiment is presented. In this run 2 mg. each of adenine and guanine were dissolved in 3.56 ml. as described above (0.04 ml. of 1 N NaOH + 0.22 ml. of methylene glycol + 3.3 ml. of butanol-water mixture) and put on the column. The cuts of the effluent were made with a time interval of 1 hour. As can be seen, a complete separation of adenine and guanine was achieved by the passage of 120 ml. of solvent through the column, which in this case corresponds to 12 hours. The yield was about 80 to 90 per cent for both adenine and guanine.

Test of Purity—The nitrogen content is a rather unsatisfactory criterion, because these values are very close for adenine and guanine and even a large contamination of the one by the other will cause only a rather slight change in the nitrogen content of the preparation. Measurement of the light absorption was considered a more rigid test of purity because of the large differences in the absorption curves of adenine and guanine. The light absorption was measured at 262 and 248 mμ in *1 N hydrochloric acid.* The quotient E_{262}/E_{248} for adenine is 1.38 and for guanine 0.69. The values of E_{262} per microgram of N per ml. and E_{248} per microgram of N per ml., determined on adenine (E_{262}) and guanine (E_{248}) isolated from polynucleotides of tissues, were found to be in good agreement with the determinations on pure adenine and guanine.

SUMMARY

A method is described for the complete and rapid separation of adenine and guanine by means of partition chromatography on starch. The method has been successfully adapted to a procedure for preparing pure specimens of adenine and guanine from comparatively small quantities of polynucleotides.

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A SYNTHESIS OF ALANINE LABELED WITH HEAVY CARBON IN THE α POSITION

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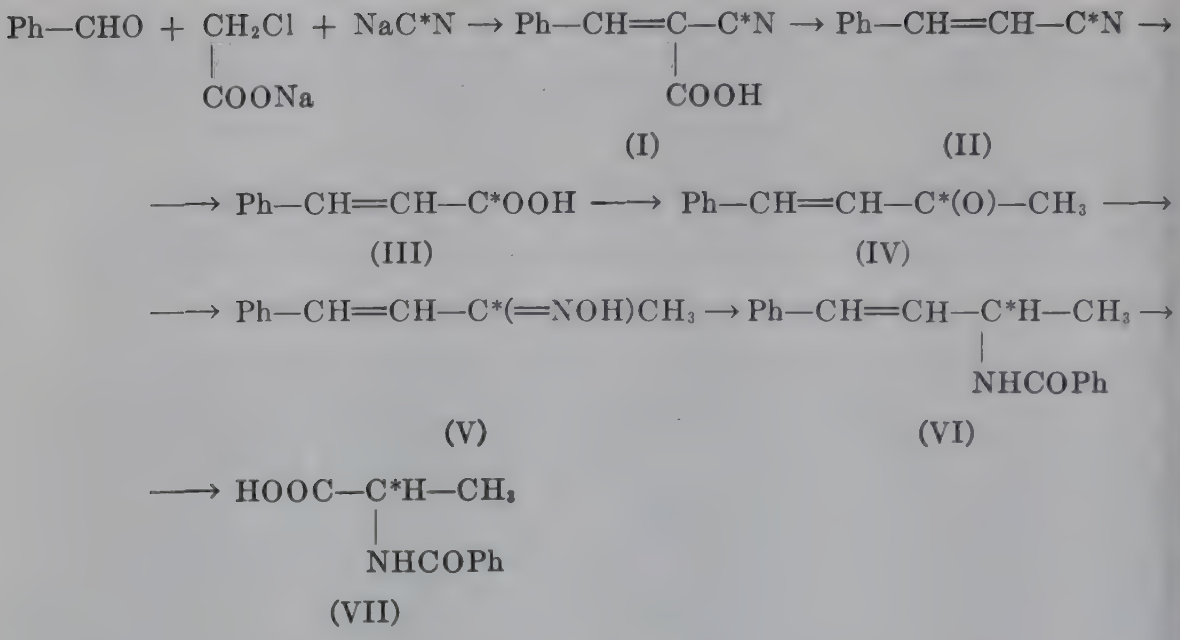
During a series of investigations on amino acid metabolism in *Torulopsis* yeast (1, 2) we required quantities of D- and L-alanine isotopically marked in position 2. The well known methods for obtaining alanine, *e.g.* from acetaldehyde by the Strecker method (3–5) or from acetic acid through acetyl cyanide and pyruvic acid (6), although theoretically capable of application in our case, were found to be unsuitable in view of the large number of steps involved and the low over-all yields. A new synthesis was, therefore, clearly indicated. For various reasons it seemed desirable to form the carboxyl group by an oxidation step at as late a stage as possible in the synthesis. With this end in view several routes were explored which aimed at the preparation of compounds of the type $R'—C^*H(NH_2)CH_3$ where R = 2-furyl- or polyhydroxyphenyl. Oxidation of such compounds after appropriate protection of the amino group should give alanine derivatives labeled in position 2. This method of introducing a carboxyl group into a molecule has been described by several workers (7, 8).

In the furane series, considerable difficulty was encountered during the introduction of the isotopic carbon, originally in the form of $Zn(CN)_2$, while, in the polyhydroxyphenyl series, the extreme sensitivity to alkali proved objectionable. Consequently these routes were not pursued further.

Attention was then directed to compounds of the type $RCH{=}CH—C^*H(NH_2)CH_3$ where oxidation of the ethylenic linkage should give the desired result. A successful synthesis on these lines is shown in the accompanying diagram.

α-Cyano-β-phenylacrylic acid (I) was obtained in 82 per cent yield by treating sodium cyanide (containing excess C^{13}) with sodium chloroacetate, followed by condensation of the resulting cyanoacetate with benzaldehyde (9). Decarboxylation of (I) by heating with copper powder gave an 85 per cent yield of cinnamic nitrile (II), which was hydrolyzed in 82 per cent yield to cinnamic acid (III) when heated with sodium hydroxide solution.

The direct conversion of cinnamic acid nitrile to benzalacetone (IV) by the Grignard method gave very low yields (10). Similarily, rather low yields were obtained when cinnamoyl chloride was treated with methyl



$$\mathrm{Ph{-}CHO} + \underset{\mathrm{COONa}}{\mathrm{CH_2Cl}} + \mathrm{NaC^*N} \rightarrow \underset{\text{(I)}}{\mathrm{Ph{-}CH{=}\underset{COOH}{C}{-}C^*N}} \rightarrow \underset{\text{(II)}}{\mathrm{Ph{-}CH{=}CH{-}C^*N}} \rightarrow$$

$$\longrightarrow \underset{\text{(III)}}{\mathrm{Ph{-}CH{=}CH{-}C^*OOH}} \longrightarrow \underset{\text{(IV)}}{\mathrm{Ph{-}CH{=}CH{-}C^*(O){-}CH_3}} \longrightarrow$$

$$\longrightarrow \underset{\text{(V)}}{\mathrm{Ph{-}CH{=}CH{-}C^*({=}NOH)CH_3}} \rightarrow \underset{\text{(VI)}}{\mathrm{Ph{-}CH{=}CH{-}\underset{NHCOPh}{C^*H}{-}CH_3}} \rightarrow$$

$$\longrightarrow \underset{\text{(VII)}}{\mathrm{HOOC{-}\underset{NHCOPh}{C^*H}{-}CH_3}}$$

cadmium (*cf.* (11)) or cinnamic acid with methyl lithium (*cf.* (12)). However, application of the method of Gilman and Van Ess (13) for converting acids to the corresponding alkyl ketones by the reaction of the lithium salts with alkyl lithium proved most successful in this case. From lithium cinnamate and 1 mole of methyl lithium, benzalacetone (IV) was obtained in a yield of more than 90 per cent and in a high state of purity, fractionation being unnecessary. The oxime (V) was prepared in the usual way. The benzoylated amine (VI) was produced by reduction with zinc and benzoylation in a manner essentially similar to the method described for the preparation of the compound by Harries and de Osa (14). The yield on this two-stage process was 40 to 45 per cent. From (VI), benzoylalanine (VII) was obtained in 55 to 65 per cent yield by oxidation with calcium or barium permanganate. Resolution and hydrolysis according to the method of Pacsu and Mullen (15) gave pure D- and L-alanine, although our yields at this step were not as high as those claimed by the American workers.

With variously substituted alkyl and aryl lithium derivatives, it should be possible to apply this method to the synthesis of a variety of amino acids. Also there are possibilities of oxidizing benzalacetone directly or indirectly to pyruvic acid labeled in the keto group. These subjects are under investigation at present.

EXPERIMENTAL

α-Cyano-β-phenylacrylic Acid (I)—This substance was obtained in 82 to 85 per cent yield by the method of Lapworth and Baker (9).

Cinnamic Nitrile (II)—Fiquet (16) obtained this nitrile in unstated yield by heating (I) *in vacuo.* In our hands an 85 per cent yield of crude nitrile, containing a little water, was obtained when 100 gm. of (I) mixed with 20 gm. of copper powder were heated for about 10 minutes at 245° until carbon dioxide evolution had ceased; the residue distilled rapidly under reduced pressure.

Cinnamic Acid (III)—14 gm. of the crude nitrile (II) were refluxed with 115 ml. of 33 per cent NaOH solution and a little ethanol until evolution of ammonia had ceased. The solution was diluted with water and acidified with sulfuric acid to pH 1. Cinnamic acid was filtered off, washed with water, and dried. Yield, 82 per cent; m.p. 133°.

Benzalacetone (IV)—An etheral solution of methyl lithium was prepared according to the directions given by van Dorp and Arens (12) from lithium and methyl iodide in ether. Meanwhile, cinnamic acid (III) was dissolved in 1 mole of aqueous lithium hydroxide and evaporated to dryness. The dry salt was washed with ether and powdered before use.

The filtered solution of methyl lithium (1.2 moles) was added gradually to a suspension of 1 mole of lithium cinnamate in ether. The total volume was approximately 4 liters. A stream of dry nitrogen was passed continuously. Heat was evolved at first but the reaction was completed by refluxing the mixture on a steam bath for 6 hours, by which time the solution was quite clear. The solution was poured onto ice and the ether layer was separated, washed with water until the washings were neutral, and then dried over sodium sulfate. Evaporation of the ether left an oil which crystallized readily on cooling, being almost pure benzalacetone. Yield, 90 to 95 per cent; m.p. 40–42°.

Benzalacetone Oxime (V)—An alcoholic solution of 1 mole of benzalacetone was refluxed with a concentrated aqueous solution of 1.2 moles of hydroxylamine hydrochloride and 1.2 moles of sodium acetate for 4 hours, allowed to stand overnight at room temperature, and evaporated *in vacuo* to a small volume. Addition of water to the residue gave the crystalline oxime. This was filtered off and dried in air. Yield 97 per cent; m.p. 89°.

1-Phenyl-3-benzamido-1-butene (VII)—40 gm. of oxime (VI) in 300 ml. of absolute ethanol and 300 ml. of acetic acid were reduced at 0° with 100 gm. of zinc dust and the mixture was worked up by the method of Harries and de Osa (14). The free base, however, was not isolated but the steam distillate was benzoylated directly by the Schotten-Baumann method, giving a 40 to 45 per cent yield of (VI); m.p. 137° after recrystallization from alcohol.

*Benzoyl-*DL*-alanine (VII)*—To 2.5 gm. of benzoyl derivative (VII) dissolved in 20 ml. of pyridine were added, alternately and with stirring, 5 gm. of barium permanganate in 40 ml. of water and 5 gm. of barium hydroxide in 50 ml. of warm water. The temperature was kept at 40°.

Addition of permanganate was stopped when a faint pink color persisted in the solution. Manganese dioxide was filtered off, washed well with hot water, and the filtrate and washings were combined. The solution was warmed with a little methanol to destroy excess permanganate, carbon dioxide was passed until the solution had only a feeble alkaline reaction, and the solid was filtered off. The filtrate was evaporated to dryness *in vacuo* and the residue dissolved in 50 per cent alcohol. Barium was removed with 4 N sulfuric acid, with rhodizonic acid indicator, and the filtered solution evaporated to a small volume. Benzoic acid was removed by extraction with ether, and pure benzoyl-DL-alanine crystallized from the aqueous solution. Yield, 65 per cent; m.p. 163°, undepressed on admixture with an authentic specimen.

When the procedure was carried out on a larger scale (120 gm.) with calcium permanganate, a 55 per cent yield was obtained.

With aid of the synthesis outlined above we prepared from labeled sodium cyanide 48 gm. of benzoyl-DL-alanine labeled with heavy carbon in position 2.

SUMMARY

A synthesis of benzoyl-DL-alanine labeled with C^{13} in the α position is described.

Resolution and hydrolysis gave the corresponding pure D- and L-alanines.

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AZLACTONES

VI. THE ACID HALIDES OF *p*-TOLUENESULFONYL-, CARBOBENZOXY-, AND BENZOYL-*p*-METHOXYPHENYL-L-ALANINE*

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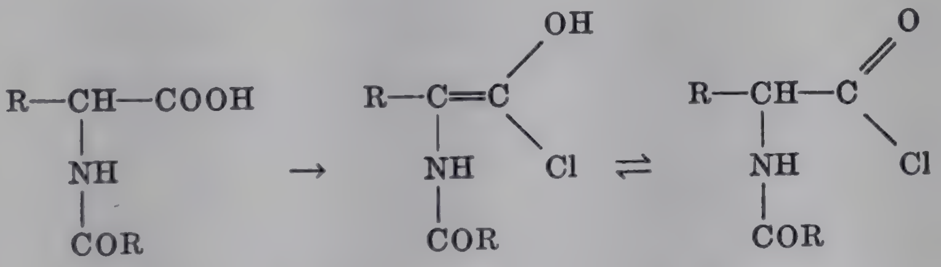
The tendency of the acid chlorides of acylamino[1] acids to racemize under very mild conditions was pointed out by Karrer and dalla Vedova (1) in 1928. To explain this optical instability they assumed the existence of the following enolization:



The fact that the acid chlorides of carbobenzoxy (2) and *p*-toluenesulfonyl (3) derivatives of amino acids retain their optical activity under conditions leading to complete racemization of acylamino acid chlorides renders this hypothesis unlikely. If the above mechanism were operative, only differences in the rate of racemization would be expected.

A more satisfactory explanation of the optical instability of acylamino acid halides was provided by observations made in the study of the chemistry of penicillin (4). In this program considerable evidence accumulated that acylamino acid halides do not have an independent existence but rearrange rapidly into the corresponding azlactone hydrohalides which racemize rapidly. The following facts support this view: (*a*) the salt-like solubility characteristics of the acylamino acid halides as compared with the covalent solubility properties of other acid halides, (*b*) the conversion of the alleged acylamino acid halides to the corresponding azlactone by simple dehydrohalogenation under anhydrous conditions or, in some cases, simply on standing *in vacuo*, (*c*) similarity of the ultraviolet absorption spectra of certain acylamino acid halides and the corresponding azlactones, (*d*) the extreme sensitivity of most acylamino acid halides toward water.[2] The following equation illustrates the mechanism suggested for this apparently spontaneous conversion of acylamino acid halide to the corresponding azlactone hydrohalide (4)

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* The material presented in this paper was taken from the thesis submitted to the Graduate College of the University of Illinois by J. W. Hinman in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

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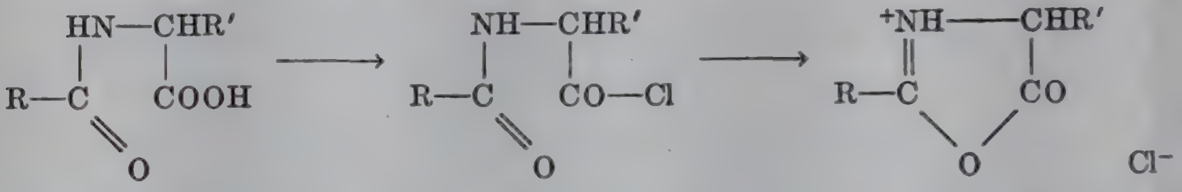
[1] In this paper acyl is used as a general term including both aroyl and alkaroyl.



The importance of acyl-, carbobenzoxy-, and *p*-toluenesulfonamido acid halides in the synthesis of peptides prompted further study of this problem with particular reference to the optical stability of the three types of compounds. In the present investigation a comparison of the acid halides of carbobenzoxy-, *p*-toluenesulfonyl-, and benzoyl-*p*-methoxyphenyl-L-alanine is made. By the use of diagnostic chemical reactions and physicochemical measurements, evidence is presented to show that the acid halides of the benzoyl derivative are actually 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone hydrohalides, and that the other two derivatives give normal acid halides of the generally accepted structures.

*Benzoyl-p-methoxyphenyl-*L*-alanine Derivatives*

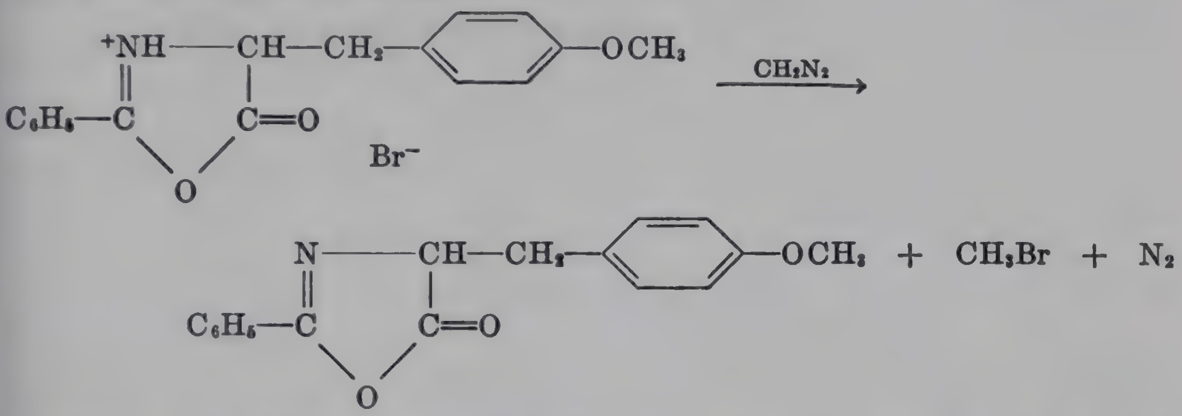
Derivatives of *p*-methoxyphenyl-L-alanine were chosen for this study because they are readily available from L-tyrosine and have reasonably high specific rotations and desirable ultraviolet absorption properties.

When an ethereal solution of benzoyl-*p*-methoxyphenyl-L-alanine was treated with phosphorus tribromide under anhydrous conditions, a crystalline, ether-insoluble salt was obtained in nearly quantitative yield. This material underwent reactions typical of both an acid bromide and an oxazolone hydrobromide. For example, treatment with water yielded benzoyl-*p*-methoxyphenyl-DL-alanine and treatment with aniline yielded benzoyl-*p*-methoxyphenyl-DL-alaninanilide. However, the reaction of the phosphorus tribromide product with diazomethane was differentiating,[3] for instead of obtaining a diazoketone, as would be the case with a true acid bromide, DL-2-phenyl-4-*p*-methoxybenzyl-5-oxazolone was ob-

[2] The acid chlorides of carbobenzoxy- and *p*-toluenesulfonamido acids are not as reactive toward water as commonly believed. Carbobenzoxyamido acid chlorides may be washed with water without deleterious results (5, 6) and the authors have observed that certain *p*-toluenesulfonamido acid chlorides persist virtually unchanged after many days in a damp atmosphere.

[3] Karrer and Bussmann (7) reported that "hippuryl chloride" yielded 2-phenyl-5-oxazolone on treatment with diazomethane.

tained. These findings indicate that the reaction proceeded, as expected, to yield the hydrobromide of the azlactone which was converted to the free azlactone with diazomethane.



However, the possibility exists that diazomethane may cause a ring closure rather than act as a simple dehydrohalogenation agent. The fact that toluenesulfon- and carbobenzoxyamido acid halides give diazoketones on treatment with diazomethane argues against such a possibility and even stronger evidence was provided by a comparison of the infrared absorption spectra of benzoyl-*p*-methoxyphenylalanine, 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone, and the phosphorus tribromide reaction product. Significant portions of the double bond region of these spectra are reproduced in Fig. 1. It will be noted that the absorption in the region 1500 to 1600 cm.$^{-1}$ characteristic of phenyl and *p*-substituted phenyl is practically the same in each of the three samples.[4] The intense *p*-substituted phenyl band at 1528 cm.$^{-1}$ in the curve for benzoyl-*p*-methoxyphenyl-L-alanine is reenforced by the second amide band. The main amide band of this compound appears at 1644 cm.$^{-1}$ and the carboxyl absorption at about 1720 cm.$^{-1}$. In Curve II for 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone, the band at 1655 cm.$^{-1}$ is attributable to the ring C=N, the 1720 cm.$^{-1}$ absorption to a trace of free carboxyl (impurity), and the band at 1832 cm.$^{-1}$ to the C=O of the azlactone ring. The spectrum of the salt-like phosphorus tribromide reaction product (Curve III) is similar to that of the free azlactone except that the ring C=O band appears at 1875 cm.$^{-1}$. A trace of free carboxyl absorption is again observable at 1720 cm.$^{-1}$. This is not surprising, considering the ease with which these compounds are hydrolyzed. The striking shift of the carbonyl absorption and absence of acid bromide type absorption in the 1820 cm.$^{-1}$ region in the spectrum of the salt-like product provide convincing evidence that this compound is not the simple acid bromide of benzoyl-*p*-methoxyphenylalanine.

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[4] The differences in the intensity of absorption are due to variation in thickness of the samples.

It seemed of interest to study the relative rates of azlactone formation and azlactone racemization. The first studies of this type were attempted by carrying out the reaction between phosphorus tribromide and benzoyl-*p*-methoxyphenyl-L-alanine in alcohol-free chloroform. However, by use of this technique, it was not possible to follow optical activity, for during the first few hours of reaction time the solution was too turbid to obtain polarimeter readings, and by the time the solution cleared the material was completely racemized.

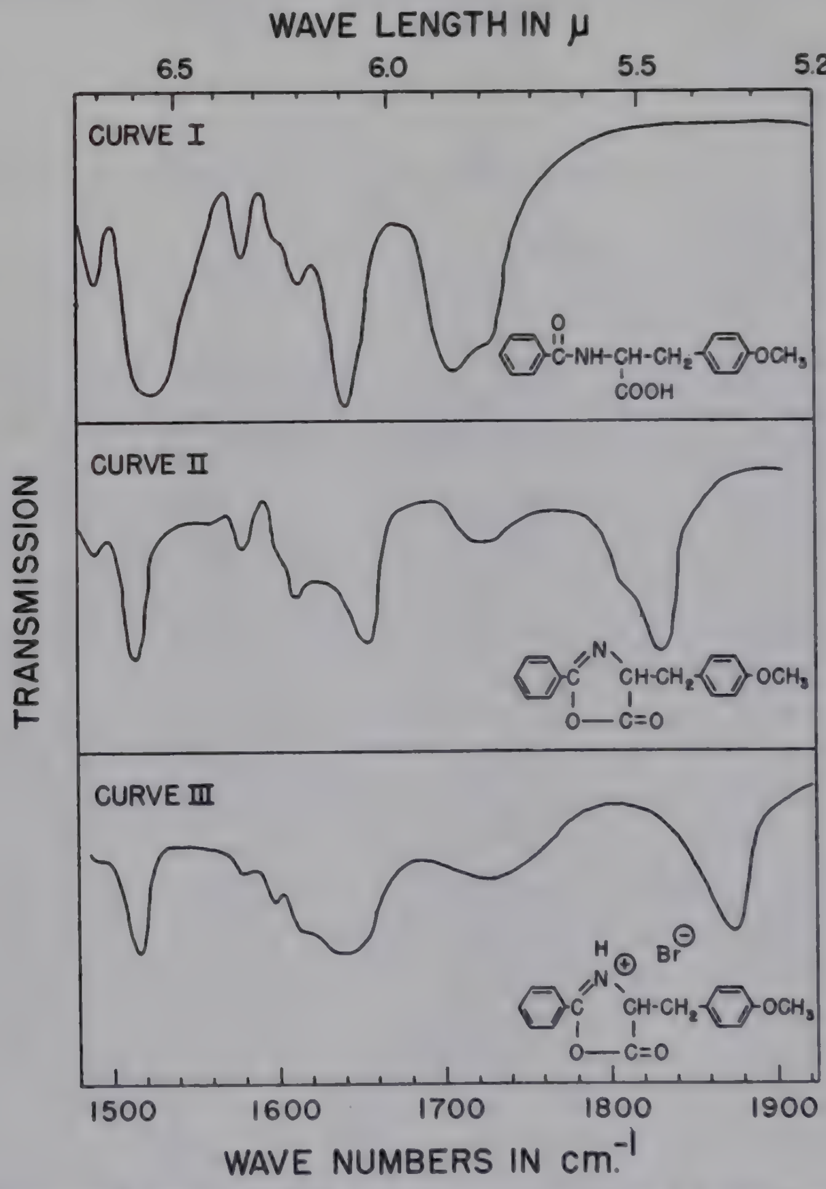

FIG. 1. Infra-red spectra of benzoyl-*p*-methoxyphenyl-L-alanine (Curve I), 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone (Curve II), and 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone hydrobromide (Curve III). Nujol mulls. Perkin-Elmer infrared spectrometer.

It was then observed that, if the chloroform used as the solvent contained a trace of ethanol, the solution remained clear during most of the reaction time, and the following changes in optical rotation were observed. Within a few minutes the rotation changed from a high positive value to a negative value, and then slowly rose to a value which, in some cases, approached the initial positive value (see "Experimental"). From such a reaction mixture, benzoyl-*p*-methoxyphenyl-L-alanine ethyl ester was isolated in good yield. The rapid drop in observed rotation probably indicated very rapid azlactonization, the azlactone being levorotatory.[5] The slow rise which followed this rapid decline in observed rotation must represent reaction of the optically active azlactone hydrobromide[6] with the ethanol present in the solution to form detrorotatory benzoyl-*p*-methoxyphenyl-L-alanine ethyl ester. Since the pure, optically active ester was isolated in nearly 70 per cent yield, the racemization reaction must proceed much more slowly than the reaction with ethanol. Essentially the same results were obtained when ether containing ethanol was used as the solvent.

Since phosphorus pentachloride has often been used for the preparation of the so called acylamino acid chlorides, a study was made of the reaction of benzoyl-*p*-methoxyphenyl-L-alanine with this halogenating agent. Surprisingly enough, when the reaction was carried out in ethereal solution, racemization was achieved within a few minutes, but in contrast to the behavior with phosphorus tribromide the solution remained perfectly clear. Concentration of the ethereal solution caused the separation of a product very similar to the hydrobromide salt except for its greater solubility in ether. Both products were exceedingly hygroscopic and lost halogen *in vacuo*. Unless great care was taken to protect the reaction mixture from even traces of moisture, the only product isolated was benzoyl-*p*-methoxyphenyl-DL-alanine. When the phosphorus pentachloride product was isolated under anhydrous conditions and allowed to react with aniline in ethereal solution, benzoyl-*p*-methoxyphenyl-DL-alaninanilide was obtained. Similarly, if the initial product was treated with ethereal diazomethane and the halogen-free reaction mixture treated with aniline, the DL-anilide was obtained.

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[5] The Squibb group (4) showed that dextrorotatory benzoyl-*p*-methoxyphenyl-L-alanine yielded on treatment with acetic anhydride the levorotatory azlactone. With acetic anhydride, approximately 2 hours were required for maximum rotation change, and after 24 hours the rotation was zero.

[6] The possibility that ethanol reacted with the acid bromide rather than the azlactone hydrobromide cannot be ruled out on the basis of this experiment. However, since a great deal of independent evidence indicates that an acylamino acid halide *per se* has an extremely short half life, it would be more reasonable to expect that this rather slow reaction took place between the relatively more stable azlactone hydrobromide and ethanol.

These findings indicate that the oxazolone salt was formed, but that it either dissociated in ether or had a greater solubility in ether than the corresponding hydrobromide salt. A careful ultraviolet absorption analysis of the benzoyl-*p*-methoxyphenylalanine-phosphorus pentachloride reaction mixture provided further evidence for azlactone formation in this reaction. In Fig. 2 the curves of the absorption spectra of pure benzoyl-*p*-methoxyphenyl-L-alanine, pure 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone, and the reaction mixture of benzoyl-*p*-methoxyphenyl-L-alanine plus 1 mole of phosphorus pentachloride are reproduced. An analysis of the *K* values of the three curves indicates an azlactone content of 82 per cent in the reaction mixture.

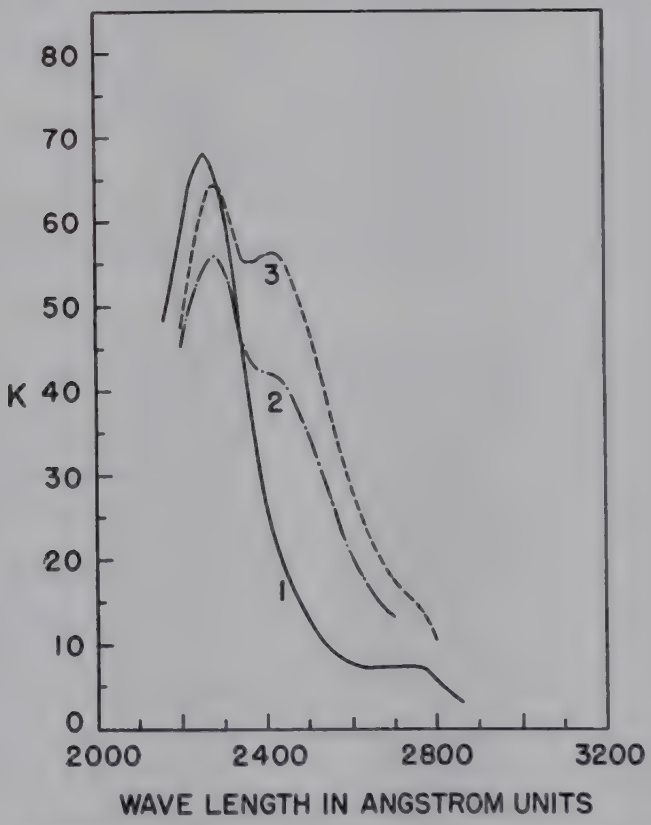
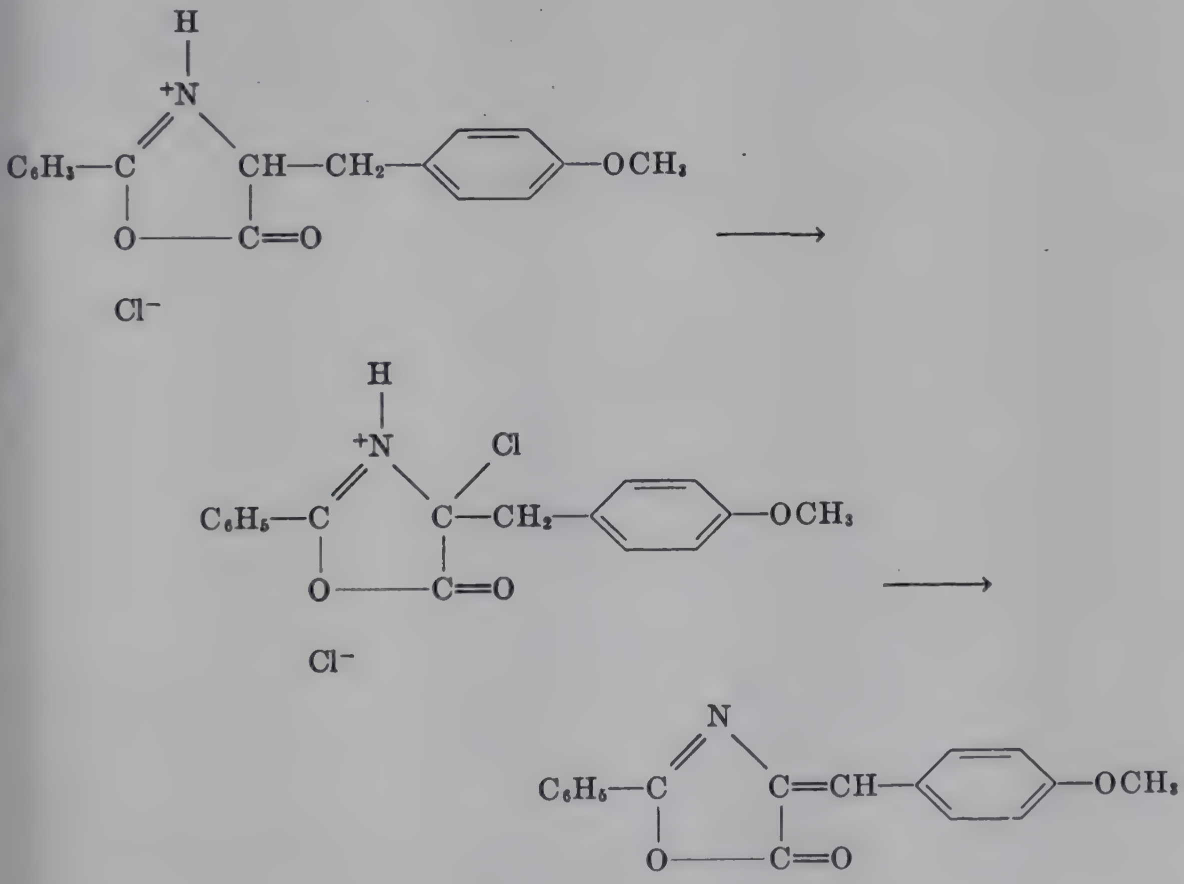


FIG. 2. Ultraviolet absorption spectra. Curve 1, benzoyl-*p*-methoxyphenyl-L-alanine; Curve 2, 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone; Curve 3, reaction mixture of benzoyl-*p*-methoxyphenyl-L-alanine plus 1 mole of PCl_5. Ether solutions. Beckman quartz spectrophotometer.

The amount of phosphorus pentachloride used in this reaction was an important factor. If less than 1 mole of the halogenating agent was used, racemization was incomplete, while addition of an excess (3 or 4 moles) caused a further reaction which was evidenced by a change in the ultraviolet absorption spectrum. The absorption maximum at 2280 A remained practically unchanged and a new peak appeared at 2550 A with a minimum at 2425 A. On working up the reaction mixture, a yellow crystalline solid was obtained. However, the spectrum of this material was still different from that of the reaction mixture. These absorption spectra are shown in Fig. 3.

The yellow crystalline solid was shown to be 2-phenyl-4-*p*-methoxybenzal-5-oxazolone by comparison with an authentic sample (8). These data seem best explained by the accompanying reactions. The oxazolone possesses an extremely active hydrogen atom which should be readily replaced by halogen. The unstable chlorine-containing intermediate may be responsible for the ultraviolet absorption spectrum of the reaction mixture, and would be expected to lose hydrogen chloride easily, yielding the unsaturated oxazolone. This method of dehydrogenating a saturated amino acid has certain advantages over that of Bergmann (9) and is being investigated further.



Carbobenzoxy-p-methoxyphenyl-L-alanine Derivatives

An attempt was made to convert carbobenzoxy-*p*-methoxyphenyl-L-alanine to its acid chloride by the action of phosphorus pentachloride in ethereal solution according to the procedure of Bovarnick and Clarke (10). With their procedure the only product obtained was the corresponding N-carboxy anhydride. However, by lowering the temperatrue and shortening the reaction time the acid chloride was obtained as a colorless crystalline solid, which readily eliminated benzylchloride giving the N-carboxy anhydride.

The stability of carbobenzoxyamido acid chlorides seems to be affected by minor changes in the procedure for their preparation, since other workers also have encountered difficulties in duplicating procedures described in the literature. In our experience carbobenzoxyamido acid chlorides should be prepared at low temperatures and used immediately.

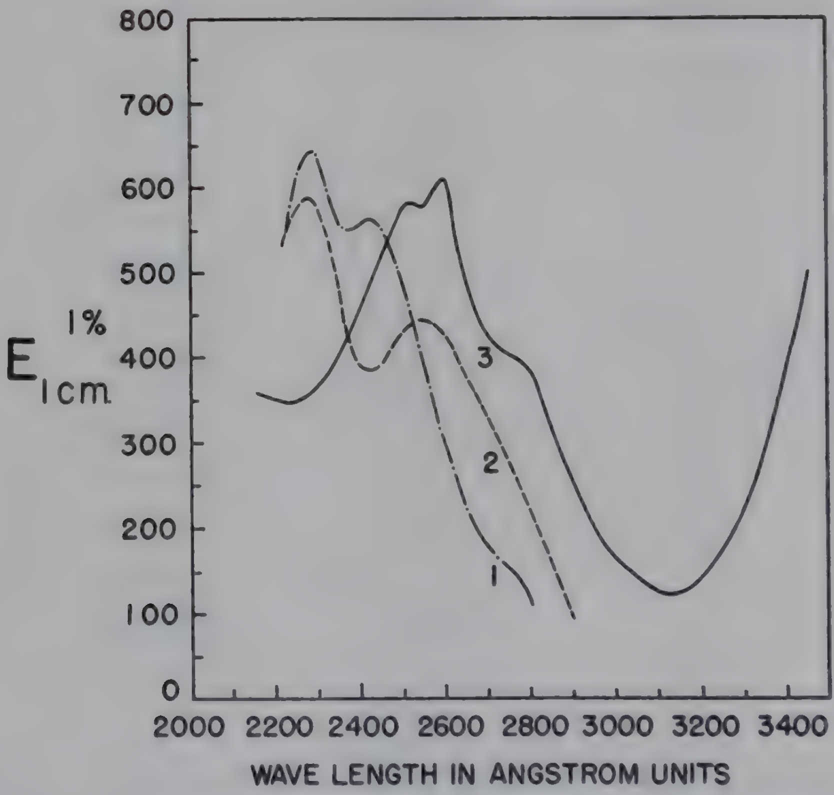


FIG. 3. Curve 1, 2-phenyl-4-*p*-methoxybenzyl-5-oxazolone (ether solution); Curve 2, reaction mixture of benzoyl-*p*-methoxyphenyl-L-alanine plus 3 to 4 moles of PCl_5 (ether solution); Curve 3, the isolated product, 2-phenyl-4-*p*-methoxybenzal-5-oxazolone (95 per cent ethanol solution).

The acid chloride reacted with aniline to yield the optically active anilide, the properties of which were in good agreement with those reported by Bovarnick and Clarke (10). In the reaction with diazomethane, carbobenzoxy-*p*-methoxyphenyl-L-alanyl chloride yielded a yellow diazoketone which, in turn, was converted to the corresponding chloroketone by the action of anhydrous hydrogen chloride. These compounds were obtained as optically active crystalline solids. No abnormal reactions were encountered and the ultraviolet absorption spectra (Fig. 4) are in keeping with the structures assigned.

*p-Toluenesulfonyl-p-methoxyphenyl-*L*-alanine Derivatives*

p-Toluenesulfonyl-*p*-methoxyphenyl-L-alanyl chloride was prepared from the acid and phosphorus pentachloride in excellent yield according to the procedure of Bovarnick and Clarke (10). The crystalline acid chloride reacted readily with methylaniline to yield the optically active methylanilide. Upon treatment with diazomethane the acid chloride yielded a crystalline diazoketone which was smoothly converted to the chloroketone by the action of hydrogen chloride gas. Optical activity was retained throughout the series of reactions. *p*-Toluenesulfonyl-*p*-methoxyphenyl-L-alanine was also converted to the acid bromide by the action of phosphorus tribromide. Addition of ethereal diazomethane to an ethereal solution of the acid bromide yielded the corresponding optically active bromoketone without isolation of the intermediate diazoketone. The infra-red absorption of *p*-toluenesulfonyl-*p*-methoxyphenyl-L-alanyl chloride was checked and found to contain the expected acid chloride band at about 1820 cm.$^{-1}$. It was of interest to note that this acid chloride was not especially sensitive to water, and it could be kept

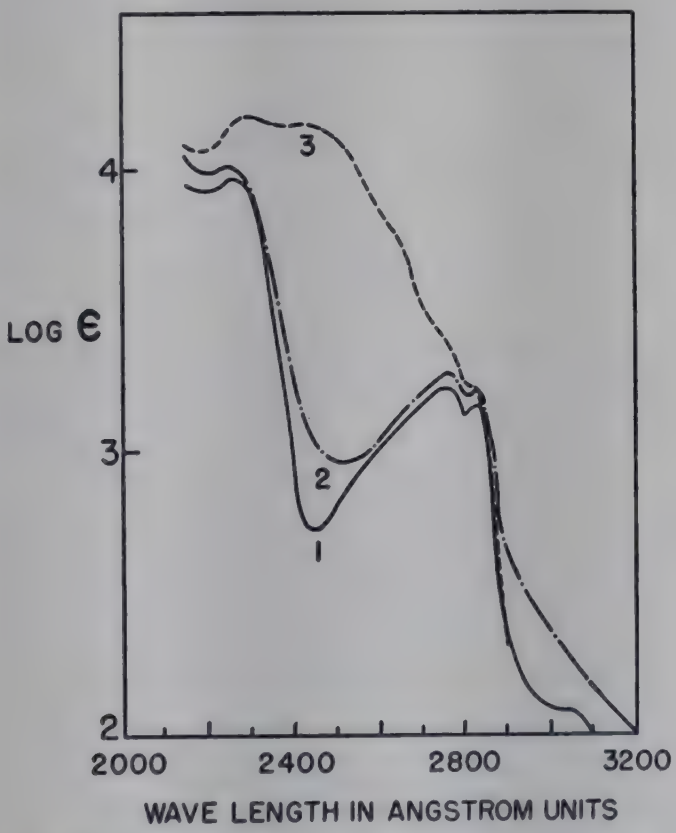


FIG. 4. Curve 1, Carbobenzoxy-*p*-methoxyphenyl-L-alanine; Curve 2, carbobenzoxy-*p*-methoxyphenyl-L-alanyl chloromethane; Curve 3, carbobenzoxy-*p*-methoxyphenyl-L-alaninanilide. 95 per cent ethanol solutions.

for several months in a vacuum desiccator without appreciable deterioration.

EXPERIMENTAL

Preparation of Derivative of p-Methoxyphenyl-L-alanine

Benzoyl-p-methoxyphenyl-L-alanine—This compound was prepared according to the procedure of Stevens (11) by benzoylation of L-tyrosine and methylation of benzoyl-L-tyrosine with dimethylsulfate in alkaline solution. The final product was recrystallized from hot water and benzene. The pure benzoyl-*p*-methoxyphenyl-L-alanine was obtained as fine colorless needles which melted at 135° (micro block).[7] The specific rotation[8] was determined at several different concentrations and in several solvents: $[\alpha]_D^{25} = -25.2°$ (2.525 per cent in 50 per cent acetic acid); $[\alpha]_D^{27} = +123.7°$ (0.38 per cent in ether); $[\alpha]_D^{25} = +124.8°$ (0.56 per cent in chloroform); $+107.8°$ (1.011 per cent in chloroform).

$C_{17}H_{17}NO_4$ (299.3). Calculated, N 4.68; found, N 4.83

DL-*2-Phenyl-4-p-methoxybenzyl-5-oxazolone* — Benzoyl-*p*-methoxyphenyl-L-alanine was azlactonized with acetic anhydride according to the method of Mohr and Stroscheim (12). After recrystallization from warm Skellysolve D (boiling range, 90–100°), the azlactone melted at 81–82°. Carter and Stevens (13) reported a melting point of 80–82° (capillary). The ultraviolet absorption spectrum of this azlactone in ether solution is characterized by two peaks, 2292 A (ϵ 18,000) and 2410 A (ϵ 16,000).

$C_{17}H_{15}NO_3$ (281.3). Calculated, N 4.98; found, N 4.97

Benzoyl-p-methoxyphenyl-L-alanine Ethyl Ester—Benzoyl-*p*-methoxyphenyl-L-alanine (504.1 mg., 1.72 mM) was dissolved in 50 ml. of magnesium-dried ethanol. The solution was cooled in an ice bath and a stream of hydrogen chloride gas was passed into the solution for 10 minutes. The temperature was allowed to rise to room temperature, and after about 20 hours the solution was concentrated to dryness *in vacuo*. The residue was dissolved in chloroform and the solution extracted with 6 per cent sodium bicarbonate solution. After washing the extract with water, and clarifying the solution by passing it through a pad of magnesium sulfate, the chloroform was evaporated. The oily residue soon crystallized and was recrystallized from ethanol-water. The slightly yellow crystals weighed 414.6 mg. and melted at 90–92°. This material was recrystallized twice from ethanol-water and the slight color removed by treatment with Darco G-60. The yield of pure white crystals melting at 92–92.5° was 374.5 mg. (66.8 per cent). $[\alpha]_D^{27} = +103.3°$ (0.663 per cent in chloroform).

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[7] All melting points recorded here are uncorrected and were taken on a micro hot stage equipped with a low power microscope.

[8] All the optical rotation data reported were determined with the use of a 1 dm. all-glass polarimeter tube. Concentrations are expressed as gm. per 100 ml. of solution.

$C_{19}H_{21}NO_4$ (327.4). Calculated, N 4.28; found, N 4.43

*Benzoyl-p-methoxyphenyl-*DL*-alanine Ethyl Ester*—This ester was prepared from benzoyl-*p*-methoxyphenyl-DL-alanine by the same procedure as given above for the L isomer. After three recrystallizations from ethanol-water the racemic ester was obtained as fine colorless needles; m.p. 90–90.5°.

*Carbobenzoxy-p-methoxyphenyl-*L*-alanine*—This compound was prepared as described by Stevens (11). The colorless crystals melted at 111–112°. $[\alpha]_D^{26} = +13.9°$ (1.514 per cent in 95 per cent ethanol).

$C_{18}H_{19}NO_5$ (329.3). Calculated, N 4.25; found, N 4.24

*Carbobenzoxy-p-methoxyphenyl-*L*-alanyl Chloride*—(*a*) Under anhydrous conditions, a mixture of 4.5 gm. (0.0137 mole) of carbobenzoxy-*p*-methoxyphenyl-L-alanine and 3.6 gm. (0.0173 mole) of phosphorus pentachloride in approximately 150 ml. of Grignard-dried ether was cooled to −5°. The mixture was shaken from time to time and then returned to the cooling bath. After 2.5 hours, most of the solid had dissolved and the bath had warmed to about 10°. After 3 hours some crystalline material had settled from the solution. This material was filtered and proved to be the N-carboxy anhydride (0.86 gm.). While the solution was still cold, 150 ml. of Skellysolve C were added and the mixture concentrated *in vacuo*. Within a few minutes a copious crystalline precipitate formed. This was collected, washed well with Skellysolve C, and dried in a vacuum desiccator. This material (1.71 gm.) was immediately soluble in ether and ethanol. The ethanol solution gave an immediate precipitate with aqueous silver nitrate. On the micro block, the colorless crystals became opaque at about 80°, but decomposed, like the N-carboxy anhydride, at 170–180° without giving a clear melt. The acid chloride could not be kept for more than a few hours at room temperature before it passed to the N-carboxy anhydride.

(*b*) In an attempt to repeat the preparation of Bovarnick and Clarke (10), only the N-carboxy anhydride was obtained. Carefully dried carbobenzoxy-*p*-methoxyphenyl-L-alanine (4.50 gm., 0.0137 mole) was dissolved in approximately 150 ml. of Grignard-dried ether. 3.6 gm. (0.0173 mole) of finely pulverized phosphorus pentachloride were added and the reaction mixture allowed to stand at room temperature for 4 hours with occasional shaking. At the end of this time the solution con tained some crystalline material. This was removed by filtration an the filtrate concentrated *in vacuo*. Four crops of crystalline solid wer collected as the solvent was removed by distillation to give a total yiel of 2.57 gm. (85 per cent). This material contained no halogen and de composed at 170–180° on the micro block without giving a clear mel For analysis the material was recrystallized from ethyl acetate. Th dense colorless crystals obtained were insoluble in ether, but soluble i ethanol.

$C_{11}H_{11}NO_4$.	Calculated.	C 59.72, H 5.02, N 6.32
(221.2)	Found.	" 60.11, " 5.33, " 6.13

*p-Toluenesulfonyl-p-methoxyphenyl-*L*-alanine*—This material was pr pared as described by Stevens (11). M.p. 139–141°; $[\alpha]_D^{26} = -20.$ (2.0 per cent in 50 per cent acetic acid).

$C_{17}H_{19}NO_5S$ (349.4). Calculated, N 4.01; found, N 4.16

*p-Toluenesulfonyl-p-methoxyphenyl-*L*-alanyl Chloride*—According to th procedure of Bovarnick and Clarke (10), a mixture of 5.50 gm. (0.01 mole) of *p*-toluenesulfonyl-*p*-methoxyphenyl-L-alanine and 3.6 gm. (0.01 mole) of phosphorus pentachloride in 200 ml. of Grignard-dried eth was allowed to stand at room temperature for 4 hours. The solutio was filtered through a carefully dried, sintered glass funnel and conce trated *in vacuo*. The colorless crystals which separated were collecte in several crops, washed with redistilled Skellysolve B, and dried in vacuum desiccator. The total yield of fine, rectangular plates meltin at 93–94° was 5.56 gm. or 96.5 per cent. Bovarnick and Clarke (1 gave no physical constants for this compound. When analyzed as Nujol mull, this material was found to have a typical acid chloride bar at about 1820 cm.$^{-1}$ in the infra-red. $[\alpha]_D^{26} = +4.63°$ (1.156 per cent chloroform).

$C_{17}H_{18}NO_4SCl$ (367.8). Calculated, N 3.81; found, N 3.79

*Reactions of Benzoyl-p-methoxyphenyl-*L*-alanine with Phosphorus Halid*

DL*-2-Phenyl-4-p-methoxybenzyl-5-oxazolone Hydrobromide*—Benzoyl- methoxyphenyl-L-alanine (1.00 gm., 3.34 mM) was weighed into a stan ard taper Erlenmeyer flask and dried for 1 to 2 hours in a vacuum ov at 50°. 150 to 200 ml. of ether were distilled from a mixture of sodiur dried ether and methylmagnesium iodide into the flask. The flask w stoppered and shaken until all of the crystals dissolved. Phosphor tribromide[9] (0.91 gm., 3.34 mM as 9.2 ml. of an ethereal solution contai ing 99.5 mg. of phosphorus tribromide per ml.) was added to the solution. Within a few minutes the solution became turbid and began to deposit a crystalline precipitate. After standing 2 to 3 hours the solid was collected in an atmosphere of dry nitrogen, washed thoroughly with Grignard-dried ether, and dried in a vacuum desiccator over phosphorus pentoxide to yield 1.20 gm. (99 per cent) of colorless, microcrystalline product, melting at 92–97°. On standing in the desiccator at room temperature the material gradually developed a yellow to tan color. On exposure to atmospheric moisture, it rapidly became sticky.

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[9] For this work, Eastman's white label phosphorus tribromide was distilled a stored in sealed ampuls.

*Conversion to Benzoyl-p-methoxyphenyl-*DL*-alanine*—250 mg. (0.71 mM) of the hydrobromide salt were added to 20 ml. of water at room temperature. The solid soon became gummy, but did not dissolve. The supernatant was decanted and fresh distilled water added. After standing overnight, the water was again decanted and 95 per cent ethanol added. The sticky solid became friable and most of it dissolved. The solution was concentrated in a stream of nitrogen and a little water added. Several hours later the crystals were collected, washed with water, and dried. The yield of benzoyl-*p*-methoxyphenyl-DL-alanine melting at 178–179° was 169.9 mg. (80 per cent). $[\alpha]_D = 0$ (0.495 per cent in chloroform).

*Conversion to Benzoyl-p-methoxyphenyl-*DL*-alaninanilide*—1 ml. of aniline was added to a suspension of 250 mg. (0.71 mM) of DL-2-phenyl-4-*p*-methoxybenzyl-5-oxazolone hydrobromide in 35 ml. of dry ether. The mixture was shaken for a few minutes and then allowed to stand overnight at room temperature. The solid was collected, washed with dilute hydrochloric acid, water, sodium bicarbonate solution, and finally with water. After drying in the vacuum oven, the colorless crystals melted at 212–214° and weighed 190.8 mg. (72 per cent). The melting point was not depressed on admixture with an authentic sample of benzoyl-*p*-methoxyphenyl-DL-alaninanilide.[10] $[\alpha]_D^{25} = 0$ (0.319 per cent in chloroform).

$C_{23}H_{22}N_2O_3$ (374.4). Calculated, N 7.48; found, N 7.27

Reaction with Diazomethane—The hydrobromide salt (1 gm., 2.76 mM) was suspended in dry ether and treated with an excess of ethereal diazomethane. After the vigorous evolution of nitrogen ceased, the solution was filtered free of a small amount of insoluble material and concentrated to dryness *in vacuo*. The residue was dissolved in approximately 40 ml. of warm Skellysolve D (boiling range, 90–100°); the solution was filtered, allowed to cool slowly to room temperature, and then cooled in the refrigerator overnight. A small seed crystal was scratched int the oil which separated. On cooling at dry ice temperature for a fe hours, most of the oil crystallized, yielding 0.45 gm. (58 per cent) of dens pale yellow crystals of the free azlactone. This material melted at 80 81° alone and at 80–82° when mixed with a sample of DL-2-phenyl-4-p methoxybenzyl-5-oxazolone prepared by the acetic anhydride metho $[\alpha]_D = 0$ in hexane solution.

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[10] On the micro block these crystals appear as transparent elongated plates which become opaque at 170–180°, begin to sublime at about 200°, and finally melt at 214–215°. Some of the crystals change from plates to long fine needles just before melting.

*Racemization of Benzoyl-p-methoxyphenyl-*L*-alanine in Alcohol-Fr Chloroform*—Benzoyl-*p*-methoxyphenyl-L-alanine (102.9 mg., 0.344 mM ($[\alpha]_D^{25} = +105.4°$ (1.03 per cent in chloroform)) was weighed into a 1 ml. volumetric flask and dried in a vacuum oven at 50° for 2 hours. Th crystals were dissolved in ethanol-free chloroform,[11] 93 mg. (0.34 mM of phosphorus tribromide added, and the solution made up to 10 ml. wit ethanol-free chloroform. The solution became turbid immediately, s that the rotation changes could not be followed. After 15 hours th solution was yellow but clear. It was optically inactive. The solutic was concentrated *in vacuo* to a gum which solidified on treatment wit water. After recrystallization from ethanol-water, 86.8 mg. (84.5 pe cent) of benzoyl-*p*-methoxyphenyl-DL-alanine of melting point 178 179° were obtained.

*Reaction of Benzoyl-p-methoxyphenyl-*L*-alanine With 1 Mole of Pho phorus Tribromide in Chloroform Containing Ethanol*—By means of th same technique as employed in the previous experiment, 100.5 mg. (0.33 mM) of benzoyl-*p*-methoxyphenyl-L-alanine were allowed to react wit 93 mg. (0.34 mM) of phosphorus tribromide in 10 ml. of Merck's reage grade chloroform (about 0.7 per cent ethanol). The changes in th observed rotation are presented in Table I.

After 54 hours, the solution was concentrated to dryness *in vacuo* ar a few drops of water added to the oily residue. When the gum had s lidified, it was collected and washed with sodium bicarbonate solutic and water. The material was then recrystallized twice from ethanc water, yielding 73.6 mg. (66.7 per cent) of beautiful colorless needl which melted at 92.5–93° alone and on admixture with benzoyl-*p*-m thoxyphenyl-L-alanine ethyl ester. The ultraviolet absorption spectru showed maxima at 2260 A ($E^{1\%}_{1\,\text{cm.}}$ 685) and at 2740 A ($E^{1\%}_{1\,\text{cm.}}$ 70) with shoulder at 2820 A ($E^{1\%}_{1\,\text{cm.}}$ 50). $[\alpha]_D^{30} = +101.6°$ (0.662 per ce in chloroform).

$C_{19}H_{21}NO_4$. Calculated. C 69.70, H 6.47, N 4.28
(327.4) Found. " 69.78, " 6.27, " 4.24

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[11] Reagent grade chloroform was shaken with concentrated sulfuric acid, wash with water, dried over calcium chloride, and fractionated from fresh calcium chlori through a 30 cm. Berl Saddles column.

When absolute ether containing a little ethanol was used as a solvent in place of the reagent grade chloroform, the results were essentially the same. The optically active ester was obtained in approximately 70 per cent yield.

*Reaction of Benzoyl-p-methoxyphenyl-*L*-alanine with 1 Mole of Phosphorus Pentachloride in Ether*—Benzoyl-*p*-methoxyphenyl-L-alanine (1 gm., 3.34 mM) was dried for an hour in a vacuum oven at 50°. Grignard-dried ether (150 ml.) was distilled into the flask and 0.70 gm. (3.38 mM) of finely ground phosphorus pentachloride was added. The mixture was shaken until all the solids dissolved. After about 10 minutes a sample of the solution was found to have zero rotation. The reaction mixture remained perfectly clear. After 2.5 hours the solution was concentrated *in vacuo* and white crystals began to form. Several crops of this material were collected, washed with Skellysolve B, and dried *in vacuo*. The yield of hydrochloride salt was 0.93 gm. or 88 per cent. The material melted at 60–70°, then resolidified in rosettes which melted at 166–167°. The freshly prepared salt was soluble in ether and slightly soluble in water. Treatment of a dilute aqueous solution of this material with aqueous silver nitrate yielded a copious precipitate of silver chloride. However, after standing overnight the material was insoluble in ether and water, contained no halogen, and melted at 179–180° (after recrystallization from benzene). In the open air, the salt reacted with atmospheric moisture and was converted to benzoyl-*p*-methoxyphenyl-DL-alanine.

TABLE I

*Optical Rotation of Benzoyl-p-methoxyphenyl-*L*-alanine Reaction with Phosphorus Tribromide in Chloroform*

Time	Observed rotation	Time	Observed rotation	Time	Observed rotation
min.	*degrees*	*min.*	*degrees*	*min.*	*degrees*
0	+1.0 *ca.*	35	+0.37	70	+0.59
3	−0.02	37	+0.39	75	+0.61
6	+0.04	40	+0.41	80	+0.61 (Solution turbid)
10	+0.09	45	+0.45	85	Too turbid to read
15	+0.15	50	+0.49	*hrs.*	
20	+0.22	55	+0.51	6	+0.62 (Solution turbid)
25	+0.27	60	+0.53	25	+0.96
30	+0.34	65	+0.56	54	+1.00

A solution of 200 mg. (0.63 mM) of the freshly prepared salt dissolved in 20 ml. of dry ether was treated with 1.0 ml. of aniline. The solution became turbid immediately. After standing overnight, the solid was collected and washed with water, dilute hydrochloric acid, sodium bicarbonate solution, and finally with water. The slightly colored, crystalline product weighed 140 mg. (59.4 per cent). After recrystallizatio from acetone-methylcyclohexane, the colorless rods became opaque a 120–140°, started to sublime at 205°, and melted sharply at 215°. Th product was thus identified as benzoyl-*p*-methoxyphenyl-DL-alaninanilide

For the ultraviolet absorption analysis, a reaction mixture containin 100.2 mg. (0.335 mM) of benzoyl-*p*-methoxyphenyl-L-alanine and 73 mg (0.35 mM) of phosphorus pentachloride in 10 ml. of Grignard-dried ethe was used. 15 minutes after the reaction was started, an aliquot wa removed and diluted with Grignard-dried ether. The curve obtaine was very similar to that of 2-phenyl-4-*p*-methoxybenzyl-5-oxazolon Analysis of the *K* values indicated the presence of about 82 per cent the azlactone in the reaction mixture.

Reaction of Benzoyl-p-methoxyphenyl-L-alanine with Excess of Pho phorus Pentachloride in Ether—A reaction mixture prepared und anhydrous conditions and containing 50.0 mg. (0.167 mM) of benzoyl- methoxyphenyl-L-alanine and 75 mg. (0.38 mM) of phosphorus pent chloride in 10 ml. of Grignard-dried ether was subjected to ultraviol absorption analysis. 30 minutes after the reaction was initiated, th absorption curve showed maxima at 2280 A and at 2550 A.

For the isolation of the product indicated by the absorption spectru 300 mg. (1 mM) of benzoyl-*p*-methoxyphenyl-L-alanine were dissolve in 60 ml. of Grignard-dried ether and treated with 0.8 gm. (about 4 m of phosphorus pentachloride. After standing at room temperature f 2.5 hours with occasional shaking, most of the phosphorus pentachlori dissolved and the yellow solution was taken to dryness *in vacuo*. T residue was taken up in fresh ether; the solution was extracted with sat rated sodium bicarbonate solution and with water. After drying ov magnesium sulfate, the ether was evaporated, leaving a quantitati yield of fine yellow crystals which were characterized as 2-phenyl-4- methoxybenzal-5-oxazolone. After recrystallization from ether or eth acetate the crystals melted at 158–159° alone and on admixture with authentic sample of the azlactone prepared from hippuric acid and an aldehyde (8). The ultraviolet absorption spectra (ethanol solutio of the two samples were likewise identical, showing maxima at 2510 (ϵ about 16,000) and 2595 A (ϵ about 17,000) and a broad shoulder about 2800 A (ϵ about 11,000).

$C_{17}H_{13}NO_3$.	Calculated.	C 73.11, H 4.69, N 5.02
(279.3)	Found.	" 72.99, " 4.86, " 4.92

Reactions of Carbobenzoxy-p-methoxyphenyl-L-alanyl Chloride

With Aniline—Aniline (1 ml.) was added to a solution of 0.50 gm. (1.4 mM) of the acid chloride dissolved in 25 ml. of dry ether. After standing overnight the mixture was poured into cold dilute hydrochloric acid. The precipitate was collected and washed with sodium bicarbonate solution and with water. This material was combined with a small crop of crystals obtained by evaporation of the ether and recrystallized twice from acetone-water to give 0.33 gm. (58 per cent) of shiny white platelets. On the micro block the platelets changed to needles at 160–170° and melted sharply at 177–178°. $[\alpha]_D^{30} = -5.4°$ (0.62 per cent in chloroform); $[\alpha]_D^{26} = +16.4°$ (0.79 per cent in acetone). Bovarnick and Clarke (10) reported a melting point of 171–173° and $[\alpha]_D^{25} = +22.3°$ in acetone (concentration not given).

$C_{24}H_{24}N_2O_4$ (404.5). Calculated, N 6.95; found, N 6.83

With Diazomethane—The acid chloride (1 gm., 2.88 mM) was dissolved in 20 ml. of dry ether. The solution was filtered free of a small amount of the N-carboxy anhydride and added slowly to a 4- or 5-fold excess of ethereal diazomethane. After a few minutes the solution was filtered and concentrated *in vacuo* until crystals began to form. A little methylcyclohexane was added and the mixture cooled in the refrigerator. After several hours the pale yellow crystals were collected and dried. The light color and melting point of 65–85° with gas evolution at about 90° indicated that this material was a mixture of the diazoketone and chloroketone. The material was then dissolved in ether and hydrogen chloride gas was passed into the solution until the yellow color was discharged. The ether was removed *in vacuo* and the residue recrystallized from ether-methylcyclohexane to yield 0.43 gm. (41 per cent based on acid chloride) of carbobenzoxy-p-methoxyphenyl-L-alanyl chloromethane melting at 114–115°. $[\alpha]_D^{30} = -38.8°$ (0.722 per cent in chloroform).

$C_{19}H_{20}NO_4Cl$ (361.8). Calculated, N 3.87; found, N 3.96

Reactions of p-Toluenesulfonyl-p-methoxyphenyl-L-alanyl Chloride

With Methylaniline—To a solution of 1.0 gm. (2.72 mM) of *p*-toluenesulfonyl-*p*-methoxyphenyl-L-alanyl chloride in 30 ml. of dry ether was added a solution of 5 ml. of freshly distilled methylaniline in 10 ml. of dry ether. After standing at room temperature for 48 hours, the mixture was poured into cold dilute hydrochloric acid, shaken, and the layers separated. The ether layer was washed with water, sodium bicarbonate solution, and finally with water. The solution was dried over magnesium sulfate, then concentrated *in vacuo* until crystals separated.

The flask was stoppered and allowed to stand overnight. During this time a mixture of clustered needles and very large dense plates separated. Skellysolve B was added; the crystals were collected and dried. The yield of crude product was 1.0 gm. Recrystallization from ether-Skellysolve B gave 0.92 gm. (77 per cent) of the methylanilide melting at 120–121°. $[\alpha]_D^{26} = +18.9°$ (1.05 per cent in 95 per cent ethanol). Bovarnick and Clarke (10) reported a melting point of 120° and $[\alpha]_D^{25} = +18.8°$ in 95 per cent ethanol.

$C_{24}H_{26}N_2O_4S$ (438.5). Calculated, N 6.39; found, N 6.14

With Diazomethane—A solution of 870 mg. (2.36 mM) of *p*-toluenesulfonyl-*p*-methoxyphenyl-L-alanyl chloride in 25 ml. of ether was added slowly to a 5-fold excess of ethereal diazomethane. After standing about 30 minutes, the solution was concentrated *in vacuo* until crystals of the diazoketone began to form. The solution was cooled in the refrigerator and a few drops of methylcyclohexane were added from time to time. After 3 days the bright yellow crystals of the diazoketone were collected and washed with a little dry ether. Yield 388 mg. (44 per cent). M.p. 87–88° with evolution of nitrogen at slightly higher temperatures. $[\alpha]_D^{25} = -57.6°$ (1.05 per cent in ether). Further concentration of the mother liquor from the crystals yielded a yellow oil which appeared to be the sulfonamide N-methylated product.

The crystalline diazoketone (100 mg., 0.268 mM) was dissolved in 12 ml. of ether and treated with dry hydrogen chloride until the yellow color was discharged. The solution was allowed to evaporate until a few crystals formed, then methylcyclohexane was added and the solution cooled in the refrigerator overnight. The yield of *p*-toluenesulfonyl-*p*-methoxyphenyl-L-alanyl chloromethane (colorless platelets), after being washed with ether and dried, was 68.4 mg. (64.5 per cent). M.p. 111–112°. $[\alpha]_D^{30} = +29.4°$ (0.477 per cent in chloroform).

$C_{18}H_{20}NO_4SCl$ (381.9). Calculated, N 3.67; found, N 3.76

*p-Toluenesulfonyl-p-methoxyphenyl-*L*-alanyl Bromomethane*—A mixture of 1.74 gm. (5 mM) of *p*-toluenesulfonyl-*p*-methoxyphenyl-L-alanine and 1.4 gm. (5 mM) of phosphorus tribromide in 150 ml. of Grignard-dried ether was allowed to stand for 6 hours at room temperature. A slight excess of ethereal diazomethane was then added slowly to the reaction mixture. The amount of gummy solid which separated was filtered off and the solution cooled over night in the refrigerator. An additional amount of the gummy solid separated. This was removed and the solution was allowed to stand at room temperature for 24 hours. During this time colorless crystals began to separate. The solution was then concentrated *in vacuo*, and the crystals collected. The yield was 1.76 gm. (82.5 per cent). For analysis a sample was recrystallized from ether. M.p. 91–92°. $[\alpha]_D^{25} = +11.7°$ (0.817 per cent in chloroform).

$C_{18}H_{20}NO_4SBr$ (426.3). Calculated, N 3.29; found, N 3.23

SUMMARY

A study has been made of the reactions of benzoyl, carbobenzoxy, and *p*-toluenesulfonyl derivatives of *p*-methoxyphenyl-L-alanine with phosphorus tribromide and phosphorus pentachloride. The evidence obtained shows clearly that benzoyl-*p*-methoxyphenyl-L-alanyl chloride (or bromide) exists only momentarily, rearranging rapidly into the corresponding salt of the azlactone and the latter racemizing rapidly. The carbobenzoxy and *p*-toluenesulfonyl derivatives give the normal acid halides with retention of optical configuration.

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INTERACTIONS OF ATABRINE, THIAMINE, AND COCARBOXYLASE

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The observation that salts of the alkaline earth metals reverse the inhibitory effects of atabrine in the growth of *Escherichia coli* (1) suggested, as one possibility, that the inhibitor acts by forming complexes with metal-catalyzed enzyme systems. The effect of atabrine on yeast carboxylase was therefore investigated, but the results show no evidence for complex formation involving atabrine and this metallo-enzyme. Instead, the investigation has disclosed that atabrine competitively inhibits the dephosphorylation of cocarboxylase by a yeast phosphatase and, further, that the drug inhibits the synthesis of thiamine by yeast cultures.

Methods

A batch of brewers' yeast which had been washed extensively in water, then dried and stored at room temperature, was used as the source of carboxylase. Atiozymase was prepared daily from this lot of yeast by rapidly washing 1 gm. with six successive 45 ml. portions of 0.1 M K_2HPO_4. A final washing was carried out with 25 ml. of 0.067 M KH_2PO_4. The temperature of the wash solutions was maintained at 10°.

Cocarboxylase concentrations were determined manometrically by the customary Warburg procedures. The components of the assay system and the order of the additions to the vessels were as follows: (1) Mn^{++}, 4.4×10^{-4} M; (2) standard cocarboxylase solution or sample; (3) thiamine, 10^{-4}, or atabrine, 10^{-3} M (see the text); (4) phosphate buffer, pH 6.1, 2.7×10^{-2} M; (5) atiozymase, 50 mg. in solution (4); (6) sodium pyruvate, 2.7×10^{-2} M; total volume 2.5 ml.; temperature 30°. The pyruvate was added after temperature equilibrium was attained and the CO_2 evolved was measured after 15 minutes.

Thiamine concentrations were determined by a thiochrome procedure of Papageorge and Lamar (2). Thiamine was extracted from cell suspensions by boiling for 1 minute in 0.05 N HCl.

Torula utilis, ATCC 9255, was grown in the mineral salts-glucose medium of Gray and Tatum (3). The inoculum employed was the growth from 10 ml. of 1 per cent Bacto-peptone incubated at 30° for 24 hours.

The cells were washed twice by centrifugation with saline and then added to 500 ml. of the mineral salts medium.

Results

The effect of the addition of atabrine to the carboxylase system was first investigated. Examination of Fig. 1 shows the dependence of the test system on the presence of cocarboxylase and manganese. In the absence of either of the two factors carboxylase activity was negligible. Under the conditions indicated about 0.8×10^{-8} mole of cocarboxylase and 1.1×10^{-6} mole of Mn^{++} were required to saturate the system.

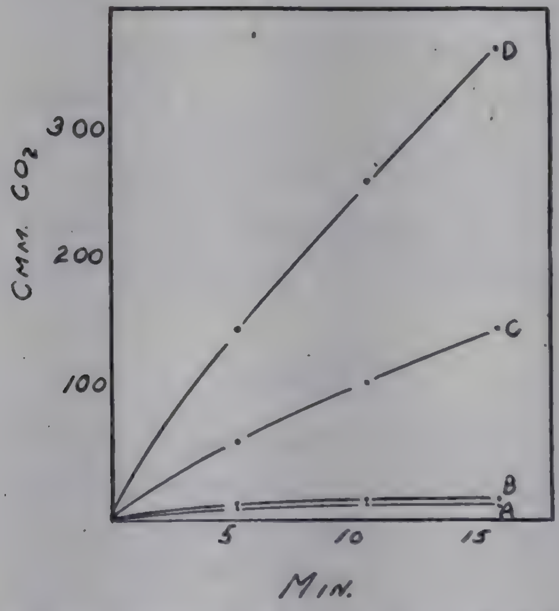

Fig. 1. Atabrine and carboxylase activity. Components of reaction mixture 100 mg. of alkaline-washed yeast; phosphate 2.7×10^{-2} M, pH 6.1; cocarboxylase 8×10^{-7} M; Mn^{++} 4.4×10^{-4} M; sodium pyruvate 2.7×10^{-2} M; volume 2.5 ml.; temperature 30°. Curve A, system lacks Mn^{++}; Curve B, system lacks cocarboxylase Curve C, complete reaction mixture; Curve D, complete reaction mixture + 8×10^{-4} M atabrine.

These points are brought out to indicate that almost complete dissociation of the enzyme had occurred during the washing procedure and thus the drug would be given a favorable opportunity to interfere with the reconstitution of the carboxylase system. However, with the addition of atabrine (8×10^{-4} M) the rate increased more than 2-fold (Fig. 1).

This stimulation of carboxylase activity by atabrine was now investigated. In the absence of added cocarboxylase, atabrine was without effect and it became apparent that the action of atabrine was similar to that of thiamine first reported by Ochoa (4). Westenbrink *et al.* (5 showed that the stimulatory effects of thiamine were due to the inhibition of a yeast phosphatase which dephosphorylates cocarboxylase.

The atiozymase preparation employed was a potent source of phosphatase. This is indicated in Tables I and II, which illustrate the rate of disappearance of cocarboxylase and the rate of formation of free thiamine from cocarboxylase by this atiozymase preparation. The initial concentration of cocarboxylase was reduced by about 50 per cent in 5 minutes.

TABLE I

Destruction of Cocarboxylase by Alkaline-Washed Yeast

Residual cocarboxylase	0 min.	5 min.	10 min.	15 min.	20 min.
A.	1.0	0.44	0.38		
B.	1.0	0.50	0.31		
C.	1.0	0.55	0.35	0.25	0.18

Values in 10^{-8} mole; alkaline-washed yeast 100 mg.; phosphate 2.7×10^{-2} M, pH 6.1; volume 2.5 ml.; temperature, 24°. The reaction was stopped at the indicated time by bringing the sample to a rapid boil over an open flame and boiling 1 minute. The supernatant solution after centrifugation was diluted for analysis.

TABLE II

Formation of Thiamine from Cocarboxylase

Thiamine	0 min.	5 min.	10 min.	15 min.	20 min.
A.	0	0.49			1.01
B.	0	0.25	0.38	0.46	

Values in 10^{-8} mole; alkaline-washed yeast 100 mg.; initial cocarboxylase 10^{-8} moles; phosphate 2.7×10^{-2} M, pH 6.1; volume 2.5 ml.; temperature, 24°.

TABLE III

Effect of Atabrine on Cocarboxylase Destruction

Residual cocarboxylase, 10^{-8} mole	Atabrine concentration			
	10^{-3} M	10^{-4} M	10^{-5} M	0 M
A.	0.35	0.15	0.07	0.07
B.	0.35	0.13	0.07	0.05

Initial cocarboxylase, 0.4×10^{-8} mole; time, 10 minutes; the other conditions are as in Table I.

The effect of atabrine on the destruction of cocarboxylase by the washed yeast preparation is indicated in Table III. With atabrine at a concentration of 10^{-3} M, the inhibition of phosphatase activity is almost complete. A slight effect is evident at 10^{-5} M under the test conditions employed.

A comparison of the effects of thiamine and atabrine on the activity of the carboxylase preparation is shown in Table IV. With the concentration of cocarboxylase employed, 4 × 10^{-7} M, maximum activity was obtained with thiamine at about 10^{-4} M; with atabrine, at about 10^{-3} M. Thus thiamine seems to be about 10 times more effective than atabrine as an inhibitor of the phosphatase activity.

TABLE IV

Comparison of Thiamine and Atabrine Effects on Carboxylase Activity

	Concentration					
	0 M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Thiamine	105	139	166	231	243	245
Atabrine			105	156	224	252

Values in c.mm. of CO_2 per 15 minutes. Alkaline-washed yeast 50 mg.; phosphate 2.7×10^{-2} M, pH 6.1; Mn^{++} 4.4×10^{-4} M; Na pyruvate 2.7×10^{-2} M; cocarboxylase 4×10^{-7} M; volume 2.5 ml.; temperature, 30°.

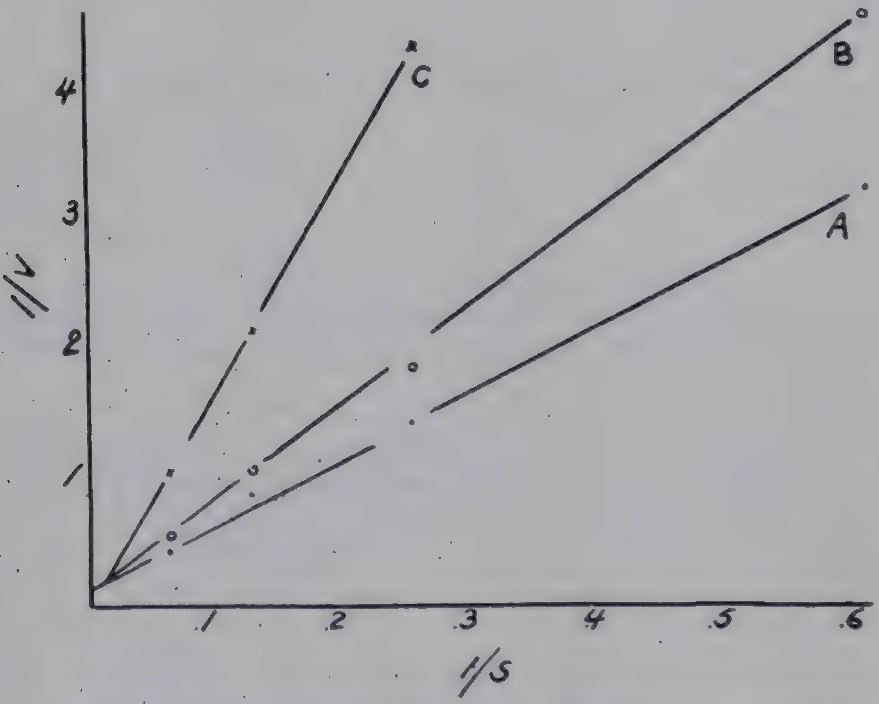

Fig. 2. Velocity of yeast phosphatase action in presence of several concentrations of cocarboxylase and atabrine. Curves A, B, and C represent mixtures containing 0, 10^{-4}, and 5 × 10^{-4} M atabrine. *S* represents molar cocarboxylase × 10^{-6} *V* represents moles × 10^{-8} cocarboxylase inactivated in 10 minutes.

The nature of the atabrine inhibition of phosphatase action appears t be that of competition. This is indicated by the data plotted in Fig. 2 The reciprocal of the reaction velocity is plotted against substrate concen ration in the presence and absence of atabrine. The progressive increase n slope with increase in concentration of atabrine, together with the pparent common intercept, indicates that the inhibition is competitive n nature (6).

The antimalarial drug, chloraquine, is more potent than atabrine as n inhibitor of the yeast phosphatase which splits cocarboxylase (Table V). Its effect was evident at a concentration of 10^{-6} M. The basic drugs, quinine, paludrine, and stilbamidine, had little or no effect. In a study of cholinesterase activity, Wright and Sabine (7) obtained somewhat similar results. They report that atabrine and chloraquine are far more effective than quinine or paludrine as inhibitors of cholinesterase. In examining Table V, it must be borne in mind that an inhibition of the cocarboxylase-cleaving mechanism is indicated by increased carboxylase activity.

Table V

Effect of Drugs on Carboxylase Activity

Concentration	Atabrine	Quinine	Paludrine	Chloraquine	Stilbamidine
M					
0	52	52	52	52	52
10^{-6}	62	52	54	93	57
10^{-5}	84	50	51	157	57
10^{-4}	178	55	45	198	45
10^{-3}	237	69	50	237	62

Values in c.mm. of CO_2 per 15 minutes. The test conditions are as in Table IV.

Massart *et al.* (8) have shown that cations can prevent the combination of basic dyes with yeast cell enzymes. However, Ca^{++}, which completely negates the action of atabrine in the growth of *Escherichia coli* (9), was without effect in reducing the toxicity of the drug for the yeast phosphatase. These negative results were obtained with Ca^{++} at a level of 6×10^{-3} M and atabrine at concentrations of 10^{-3} and 8×10^{-5} M.

Yeast carboxylase itself is relatively insensitive to the action of the antimalarial. A 20 per cent reduction of the maximum activity was observed with atabrine at a concentration of 1.2×10^{-2} M.

The effect of atabrine on yeast growth activity with respect to crop yield and thiamine and cocarboxylase cell content was next examined. The yeast *Torula utilis* was employed in these studies. This organism grows well in a mineral salts-glucose medium and its growth is markedly stimulated by the addition of thiamine. An examination of freshly harvested cells (18 hours old) grown without added thiamine showed that they contained less than 0.2 γ of free thiamine per gm. (dry basis). Thus the cellular thiamine is almost completely in the form of cocarboxylase (Table VI).

The effects of atabrine are shown in Table VI. In the absence of added thiamine, increasing concentrations of atabrine progressively inhibited the cell yield and, associated with this, a progressive decrease in cocarboxylase content occurred. In the presence of added thiamine, the crop yield was somewhat more than doubled and the cocarboxylase content increased 8- to 10-fold. Again, progressive decreases in cell yield were observed with increasing atabrine concentrations. However, no decreases in cocarboxylase content occurred. Apparently atabrine interference with thiamine metabolism in the growth of *Torula utilis* involves a step in the synthesis of thiamine and not the conversion of the latter to cocarboxylase.

TABLE VI

Effect of Atabrine on Cocarboxylase Formation and Cell Yield

	No thiamine		5×10^{-6} M thiamine	
Atabrine, M	0		0	
Yield, *ml.*	0.19		0.45	
Cocarboxylase, γ *per gm.*	35.1		322	
Atabrine, M	0	5×10^{-6}	0	5×10^{-6}
Yield, *ml.*	0.20	0.20	0.43	0.45
Cocarboxylase, γ *per gm.*	45.0	42.5	339	346
Atabrine, M	0	10^{-5}	0	10^{-5}
Yield, *ml.*	0.20	0.16	0.42	0.35
Cocarboxylase, γ *per gm.*	37	33.3	296	272
Atabrine, M	0	2×10^{-5}	0	2×10^{-5}
Yield, ml.	0.19	0.12	0.41	0.33
Cocarboxylase, γ *per gm.*	47.0	31.6	358	351
Atabrine, M	0	2×10^{-5}	0	2×10^{-5}
Yield, *ml.*	0.18	0.12	0.36	0.19
Cocarboxylase, γ *per gm.*	39.7	27.0	329	359
Atabrine, M			0	3×10^{-5}
Yield, *ml.*			0.39	0.15
Cocarboxylase, γ *per gm.*			324	317

The yields are expressed in terms of packed cell paste recovered on centrifugation from 500 ml. of mineral salts medium after 18 hours incubation at 30°. Cocarboxylase concentrations are expressed in micrograms per gm. of dry cell weight.

DISCUSSION

Because (*a*) atabrine bears some structural relationship to riboflavin and (*b*) the drug can interfere with reactions involving flavin nucleotides, some support has been given to the concept that a flavin inhibitor antagonism exists which is specific for atabrine (10–13). However, there are objections to accepting the flavin-atabrine antagonism as specific in nature. Hellerman *et al.* (14) have already pointed out some inconsistencies. The data reported now dealing with atabrine and thiamine relationships indicate that the growth-inhibitory action of atabrine is not limited to interference of systems involving flavin derivatives. In this connection it may be noted that riboflavin was no more effective than thiamine or several other B vitamins in reversing the bacteriostatic action of atabrine in the growth of *Escherichia coli* (15).

It seems unlikely that the inhibition of cocarboxylase dephosphorylation observed in dried yeast preparations plays a rôle in the inhibition of cell growth. One would expect that, if the inhibition of cocarboxylase cleavage were associated with inhibition of yeast growth, increases in cellular cocarboxylase would occur in cells whose growth was limited by the presence of atabrine. However, cells grown in the presence of atabrine have cocarboxylase concentration below or equal to that of normal cells.

Hegsted *et al.* (16) have reported that atabrine exerts a thiamine-sparing action in the growth of rats fed a thiamine-deficient diet. A somewhat similar picture is presented by the action of atabrine as it affects carboxylase activity in dried yeast. In this instance, however, atabrine exerts a "cocarboxylase sparing" action by inhibiting inactivation of the coenzyme. It may very well be that the sparing action of atabrine in the rat results from its interference with cellular mechanisms which normally convert the enzymatically active form of thiamine, diphosphothiamine, to inactive forms.

SUMMARY

1. Atabrine competitively inhibits the dephosphorylation of cocarboxylase by a dried yeast preparation. Chloraquine is a more effective inhibitor than atabrine, whereas quinine, paludrine, and stilbamidine are without effect.

2. Atabrine inhibits the synthesis of thiamine by growing cells of *Torula utilis*. At concentrations causing growth inhibition the drug is without effect on the conversion of thiamine to diphosphothiamine.

3. Thiamine in freshly harvested cells of *Torula utilis* is almost completely in the form of cocarboxylase.

4. It is concluded that the atabrine inhibition of cocarboxylase dephosphorylation observed in dried yeast preparations does not have a rôle in accounting for the growth-inhibitory effects of the drug.

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THE ULTRAVIOLET ABSORPTION SPECTRA OF THE PYRIMIDINE RIBONUCLEOSIDES AND RIBONUCLEOTIDES*

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It is well known that the high and characteristic absorption of nucleic acids in ultraviolet light is due to the absorption of their purine and pyrimidine components. Several workers have measured the absorption curves of the amino- and oxypurines (1–3) and Jordan has obtained similar data for adenosine and guanosine and for the corresponding nucleotides (3). In the latter instances substitution of the carbohydrate radical in position 9 of the purine ring does not change the absorption appreciably, but, as shown by Gulland and coworkers (4), substitution in the 7 position of either adenine or guanine causes definite changes in the absorption spectra.

Although the absorption curves of the free pyrimidines, uracil, cytosine, and thymine (2, 5, 6), and of thymine desoxyriboside (7) have been determined, similar data have not been published for the corresponding ribonucleosides, cytidine and uridine, or for the nucleotides, cytidylic acid and uridylic acid. With the growing interest in the metabolism of these compounds (8, 9), knowledge of their absorption characteristics would be useful for their determination in biological material. As it has been possible recently to prepare them in appreciable yield and of a high degree of purity (10, 11), we have accordingly measured their absorption in comparison with the free pyrimidines, cytosine and uracil. As all of these compounds may exist in different tautomeric forms, depending on the hydrogen ion concentration of the solvents employed, dilute acid and dilute alkali were used to provide conditions under which one or the other form would be present. For comparison 0.05 M phosphate buffer at pH 7.0 was also used.

EXPERIMENTAL

Materials—Synthetic samples of cytosine and uracil (12) as well as a commercial sample of uracil (Eastman Kodak Company) were used. Both uracil samples were recrystallized several additional times from water and melted with decomposition at 329–333° (when placed in a melt-

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† National Institute of Health Predoctorate Fellow, 1948.

ing point block at 315°). They were dried to constant weight *in vacuo* over phosphorus pentoxide at 100° before using. The sample of cytosine was recrystallized two additional times from water. It melted with decomposition at 315–318° and was used in the form of the monohydrate.

Cytidine, uridine, cytidylic acid, and diammonium uridylate were prepared by methods developed in this laboratory (10, 11). The first three compounds were dried to constant weight *in vacuo* over phosphorus pentoxide at 100°. The diammonium uridylate was used as the monohydrate (10).

Solutions—Stock solutions of each compound were prepared containing approximately 0.1 mM in 100 ml. of distilled water. 10 ml. aliquots were diluted to 100 ml. with 0.05 M potassium phosphate buffer at pH 7.0 for measurements at this pH or with sufficient standard hydrochloric acid or sodium hydroxide to give solutions that were 0.01 N with respect to acid or alkali.

Optical Methods—All measurements were made on a model DU Beckman spectrophotometer with the ultraviolet light attachment. Standard calibrated silica cells, 1 cm. wide with a light path of 1 cm., were used throughout. Both the test solution and the appropriate blanks were pipetted into the cells. Extinction readings were usually made with the selector-switch in the 1.0 position, but where the density values were larger than 1.0, greater accuracy was obtained by using the selector-switch in the 0.1 position. The wave-length scale was calibrated by using a mercury vapor lamp, the 253.6 and the 365.0 mμ bands being used. Density measurements on a standard potassium chromate solution (5.00×10^{-4} M anhydrous potassium chromate in 0.05 M potassium hydroxide) checked the values recorded in the literature (13).

RESULTS AND DISCUSSION

Uracil and Cytosine—The absorption curves of uracil and cytosine in the solvents mentioned above are shown in Figs. 1 and 2. The results with cytosine are in good agreement with the previously published data (6), even though slightly weaker alkali was used in the present experiments. Those with uracil show general agreement as to the wave-lengths at which maxima and minima have been found previously, but differ significantly in the value of the molecular extinction coefficient, E_M. Thus in acid or neutral solution the value for E_M may be observed to vary from about 8200 found in the present experiments for both 0.01 N hydrochloric acid and phosphate buffer at pH 7.0 to 9500 found at pH 2.6 and 6.5 (5), and to 11,000 found for a solution in a Kolthoff buffer at pH 3 (14). Whether the discrepancies are due to differences in the uracil samples, resulting possibly from their preparation under different light conditions (5), or to the methods of measurement employed is not apparent, but the two preparations used in the present experiments gave identical results within experimental error in three duplicate experiments carried out on each sample. It should be pointed out that no unusual precautions were taken to avoid exposure of the crystalline samples to light other than to avoid direct contact with sunlight.

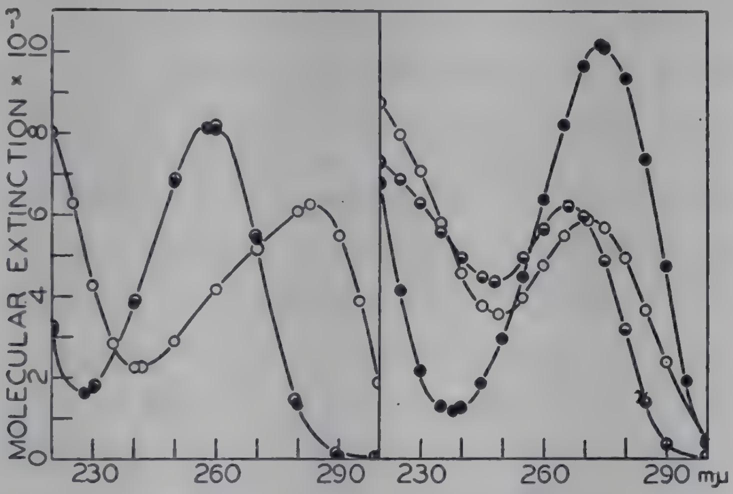

FIG. 1 FIG. 2

FIGS. 1 AND 2. Absorption curves of uracil (Fig. 1) and cytosine (Fig. 2). ○, in 0.01 N sodium hydroxide; ⊖, in 0.05 M potassium phosphate buffer at pH 7.0; and ●, in 0.01 N hydrochloric acid.

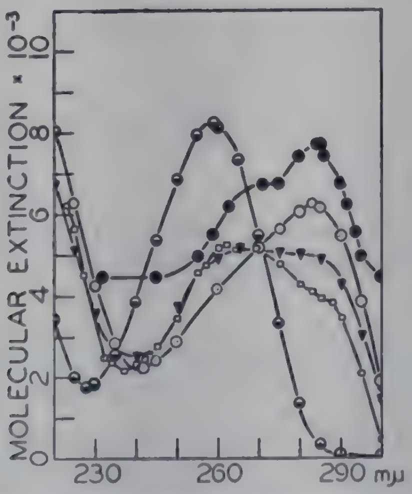

FIG. 3. Absorption curves of uracil under different conditions of alkalinity. ⊖, in 0.05 M potassium phosphate buffer at pH 7.0; □, in water at pH 9.0 to 9.5; ●, in Kolthoff buffer at pH 11 (14); and ▲, in 0.001 N, and ○, in 0.01 N sodium hydroxide.

It is of interest from the standpoint of the tautomeric equilibria concerned that both uracil and cytosine show absorption maxima near 280 mμ in alkali. The absorption curves of uracil at pH 7.0 and in 0.01 N alkali are reproduced in Fig. 3 in comparison with the absorption data found in 0.001 N alkali and with the previously published data for uracil at a pH estimated to be between 9.0 and 9.5 (5) and with data obtained in a Kolthoff buffer at pH 11 (14). The lack of agreement between our results in 0.001 N alkali at approximately the same pH as that of the Kolthoff buffer is evident. It seems likely that the increased absorption in the buffer solution is due to certain of the buffer constituents, which, however, were not given in the publication cited. It may be seen from a comparison of the other data that the value for the molecular extinction at 260 mμ observed in phosphate or in acid decreases as the solution is made alkaline and practically disappears in 0.01 N alkali concomitant with the appearance of the new maximum at 280 mμ. As the pK value of uracil is 9.45, it is evident that the appearance of the second maximum is correlated with salt formation and probably with the development of a more fully aromatic structure, as the hydrogen atom at position 1 or at position 3 is shifted to 6 or 2, respectively, under these conditions.

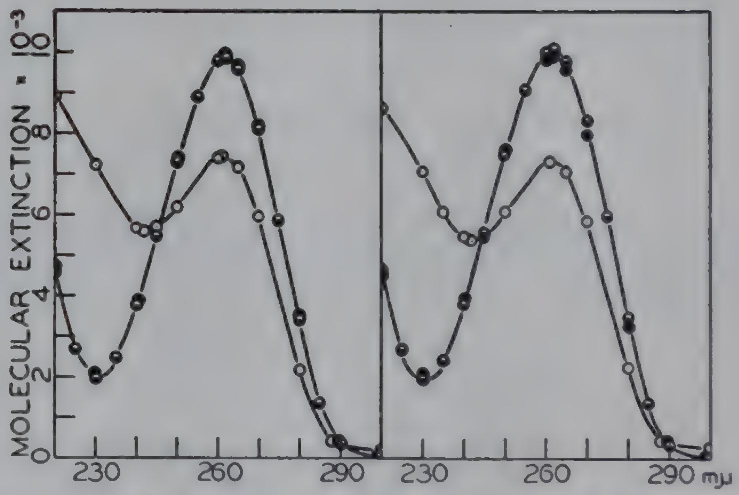

FIG. 4 FIG. 5

FIGS. 4 AND 5. Absorption curves of uridine (Fig. 4) and uridylic acid (Fig. 5). The symbols refer to the same solvents as in Fig. 1.

Uridine and Uridylic Acid—The absorption curves of uridine and uridylic acid are presented in Figs. 4 and 5. A comparison of the graphs shows that the two compounds gave almost identical results over the wave-lengths studied. Substitution of the phosphate group in the 3′ position of the ribose side chain has no appreciable effect on the pyrimidine ring structure present in uridine itself. The absorption curves for uridine and uracil, however, are significantly different. In acid or in neutral solution the curves are similar in appearance, but the absorption maximum of uridine has a value of about 9800, as compared to about 8200 for uracil. In alkali, although the maximum with uridine is decreased to 7400, there is no corresponding shift towards the longer wave-lengths. A possible explanation for the difference in the two cases is that substitution of the labile hydrogen atom in position 3 of uracil with the stable ribosidic linkage has eliminated the possibility of the fully aromatic pyrimidine ring which may be formed with either uracil or cytosine in the presence of alkali.

A comparison of the absorption curves of uridine and of thymine desoxyriboside (6) shows a striking similarity between the two under the same conditions. In acid or in alkaline solution nearly identical values for the molecular extinction coefficient are found for both compounds. The presence of the methyl group in the 5 position of the pyrimidine ring in thymine desoxyriboside, however, results in a slight shift of the maximum and minimum towards the longer wave-lengths. While no similar proof of the location of desoxyribose at the 3 position of the pyrimidine ring in thymine desoxyriboside has been offered, as in the case of uridine, the similar absorption characteristics of the two compounds in acid and in alkali provide evidence that this is actually the case. Thymine in which a hydrogen atom is present in the 3 position shows a similar shift in absorption maximum to 290 mμ in alkali as that found for uracil (7).

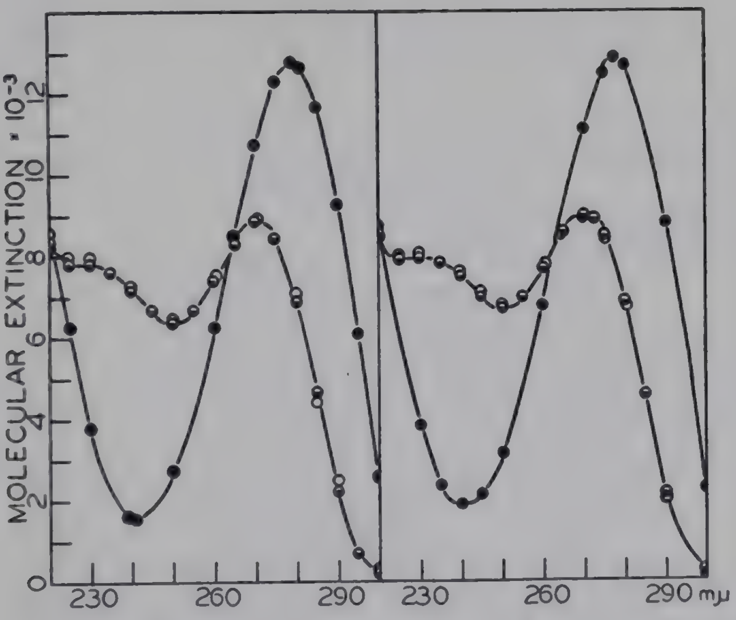

Fig. 6 Fig. 7

Figs. 6 and 7. Absorption curves of cytidine (Fig. 6) and cytidylic acid (Fig. 7). The symbols refer to the same solvents as in Fig. 1.

Cytidine and Cytidylic Acid—The absorption curves of cytidine[1] and cytidylic acid are shown in Figs. 6 and 7 and their absorption characteristics in comparison with uracil, cytosine, uridine, and uridylic acid are summarized in Table I. It may be seen that the curves for cytidine and cytidylic acid are identical within experimental error. In acid the same maximum was found at 280 mμ and the same minimum at 240 mμ, and the value for the molar extinction coefficient was approximately 12,750 in each case. Curves of similar shapes that were characteristically different from those in acid were found in both phosphate and in alkali. Under these latter conditions, as with cytosine, the absorption maxima for both compounds were also shifted to 270 mμ. Values of the molecular extinction coefficient at this wave-length were reduced to about 9000, but the differences between the results in phosphate and in alkali were not as pronounced as in the case of cytosine. At the same time the amount of absorption at 230 mμ was greatly increased, the curves showing a definite shoulder or plateau at this point in contrast to those for cytosine under the same conditions. The curves for cytidine and for cytidylic acid in alkali and in phosphate could scarcely be distinguished from each other. Because of the high absorption of cytidine and cytidylic acid at 270 to 280 mμ, it is evident that either compound may be confused with the aromatic amino acids, tyrosine and tryptophan, which also absorb strongly at these wave-lengths ((13) p. 185).

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[1] In recent publications (15) Hotchkiss and Reichard independently present absorption data for cytidine at various wave-lengths. The values given are similar but not identical to those calculated from the data found for the highly purified samples used in the present publication.

TABLE I

Absorption Characteristics of Cytosine, Uracil, Cytidine, Uridine, Cytidylic Acid, and Uridylic Acid

E_M = molar extinction coefficient.

	Potassium phosphate buffer (pH 7.0)				Sodium hydroxide (0.01 N)				Hydrochloric acid (0.01 N)			
	Maxima		Minima		Maxima		Minima		Maxima		Minima	
	mμ	E_M	*mμ*	E_M	*mμ*	E_M	*mμ*	E_M	*mμ*	E_M	*mμ*	E_M
Cytosine	266	6,200	249	4360	271	5870	249	3550	274	10,140	238	1170
Uracil	259	8,200	228	1750	284	6230	242	2220	258	8,130	228	1610
Cytidine	270	8,830	250	6360	271	8900	250	6410	279	12,750	241	1550
Uridine	262	9,820	230	1960	261	7400	242	5560	262	9,930	230	2060
Cytidylic acid	270	8,970	250	6680	272	8880	250	6780	278	12,720	240	1870
Uridylic acid	262	10,040	230	1950	261	7290	242	5370	262	9,890	230	1950

Of the three solvents employed, the largest differences in absorption between the amino and oxy compounds occurred when phosphate buffer at pH 7 was used. As the use of such a buffer provides more reproducible conditions than the use of distilled water, it would appear to be more suitable for characterization of the individual compounds. It is also apparent, however, that with uridine and uridylic acid the position of the absorption maximum remains the same, regardless of the solvent used, while in all the other cases significant shifts occur as either acid or alkali is used, and that absorption measurements in both of these solvents can be of value in the identification of the individual pyrimidine derivative.

SUMMARY

The absorption spectra of uridine and cytidine in comparison with the corresponding free pyrimidine bases, uracil and cytosine, and with the corresponding nucleotides, uridylic acid and cytidylic acid, have been determined in phosphate buffer at pH 7.0, in 0.01 N hydrochloric acid, and in 0.01 N sodium hydroxide.

The absorption curves of uridine closely resemble those for uridylic acid and are different in several respects from those for uracil or for cytidine or cytidylic acid under similar conditions. The last two compounds mentioned show very nearly the same absorption characteristics, which are also different from the free base, cytosine. Phosphate buffer at pH 7.0 is a more suitable solvent for the characterization of either the free pyrimidines or the nucleosides or nucleotides.

The close similarities between the absorption curves of uridine and thymine desoxyriboside in acid and in alkali are offered as evidence that the desoxyribose group in thymine desoxyriboside is substituted in the 3 position of thymine.

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THE DEAMINATION OF CYTIDINE IN ACID SOLUTION AND THE PREPARATION OF URIDINE AND CYTIDINE BY ACID HYDROLYSIS OF YEAST NUCLEIC ACID*

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Although acid hydrolysis of yeast ribonucleic acid has been used as a standard procedure for the preparation of the pyrimidine ribonucleotides ((1), (2) p. 221), it has not been used extensively for obtaining the corresponding nucleosides. Under extremely vigorous conditions (2 hours at 175° in 25 per cent sulfuric acid) the free pyrimidines, uracil and cytosine, have been the products isolated (3). That it is possible to prepare cytidine after hydrolysis of cytidylic acid or nucleic acid with 10 per cent sulfuric acid at 130° in a sealed tube was shown by Levene (4) and by Loring and Pierce (5) in experiments in which either cytidine was the only product isolated or the yield of cytosine was relatively small. Of the two commonly accepted procedures for the preparation of the pyrimidine nucleosides, ammoniacal hydrolysis under pressure ((6) p. 112) or refluxing with pyridine (7), the former is difficult to carry out on a preparative scale in many biochemical laboratories because of a lack of suitable high pressure equipment. The second procedure requires a relatively expensive reagent and in our laboratory has given low yields of cytidine (5).[1] In an attempt to improve the method for the preparation of these compounds, experiments were conducted to determine the usefulness of acid hydrolysis under reflux conditions as a method of obtaining cytidine and uridine.

As inorganic phosphate is slowly liberated from the pyrimidine nucleotides during acid hydrolysis ((6) p. 44), it is evident that the success of the method for the preparation of the nucleosides depends on the relative rates both of liberation of inorganic phosphate and of destruction of pyrimidine nucleosides under these conditions. The stability of cytidine and uridine to acid hydrolysis was determined by assaying solutions of known concentration for pyrimidine nucleoside activity with the pyrimidine-deficient *Neurospora* mutant 1298 after refluxing in solutions of different normalities for different lengths of time. The results showed that

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† National Institute of Health Predoctorate Fellow, 1948.

[1] Harris and Thomas (8) have isolated uridine in a yield of 12 per cent after pyridine hydrolysis of yeast nucleic acid.

pyrimidine nucleoside activity was not significantly affected after boilin for 24 to 48 hours in solutions that were 1 N or less with respect to aci but that refluxing with 6 N acid caused appreciable loss of activity after 2 hours. Experiments on the liberation of inorganic phosphate from cytidylic acid and from yeast nucleic acid in boiling 0.4 N sulfuric acid showe that approximately 48 hours were required for the complete hydrolysis t the nucleoside stage. Application of the cytidine-uridine fractionatio procedure (9) to a solution of cytidine that had been refluxed in 0.4 sulfuric acid for 48 hours provided evidence that cytidine was converte to uridine under these conditions, and this result was confirmed by spectrophotometric analysis of the hydrolysate, by estimation of the ammoni formed, and by isolation of crystalline uridine.

On the basis of the above-mentioned results, it has been possible t devise a relatively simple method for the preparation of uridine fro yeast nucleic acid. The nucleic acid is first subjected to acid hydrolysi in 0.4 N sulfuric acid for 2 hours and the liberated adenine and guanin removed by silver precipitation. After removal of excess silver, the filtrat containing the pyrimidine nucleotides is refluxed for an additional 4 hours, and the remaining cytidine and other basic substances are remove with phospho-12-tungstic acid. The excess phospho-12-tungstic acid an phosphate ions are removed by the addition of barium hydroxide to pH 8 Traces of inorganic and organic ions are removed by successive treatment with Duolite C-3 and A-2 and the resulting filtrate after evaporation to syrup crystallized by the addition of alcohol. Yields of 12 to 13 per cen uridine based on the original nucleic acid have been obtained.

After a shorter period of acid hydrolysis of yeast nucleic acid, it wa also possible to isolate cytidine nitrate in a yield of 3.1 per cent. Thi latter procedure, however, does not appear to be as advantageous for th preparation of cytidine as that given by Bredereck *et al.* (7). A simpl and convenient method for the preparation of free cytidine from cytidin nitrate by the use of Duolite A-2, however, has been developed. Th details of the several experiments mentioned above are presented in thi paper.

EXPERIMENTAL

Stability of Pyrimidine Nucleosides As Compared to Rates of Liberatio of Inorganic Phosphate from Cytidylic Acid and from Yeast Nucleic Acid— The stability of cytidine to acid hydrolysis was determined by refluxin solutions containing 0.4 mg. of cytidine per ml. in 0.1, 0.2, 1, and 5.5 N sulfuric acid and in 1 and 6 N hydrochloric acid for 24 and 48 hours. The solutions were diluted to a measured volume and assayed for pyrimidin nucleoside activity with the *Neurospora* mutant 1298 (9). The stabilit of uridine was determined similarly after refluxing 20 mg. in 50 ml. of 0.2 N and 0.4 N sulfuric acid for 48 hours. The rate of liberation of inorganic phosphate from the pyrimidine nucleotides has been determined previously ((6) p. 44; (2) p. 211). In the present experiments solutions containing 25 mg. of cytidylic acid in 50 ml. of 0.1 N and of 0.5 N hydrochloric acid were used. Aliquots were removed at the end of 24 and 48 hours, assayed for pyrimidine nucleoside activity, and analyzed for inorganic phosphate by the method of King (10). A similar experiment was also performed with yeast nucleic acid in 0.4 N sulfuric acid. Solutions containing 100 mg. in 50 ml. of acid were refluxed for 24, 48, and 72 hours and analyzed for inorganic phosphate and pyrimidine nucleoside activity.

TABLE I

Stability of Pyrimidine Nucleoside Activity to Acid Hydrolysis, As Compared to Rate of Liberation of Inorganic Phosphate from Cytidylic Acid and from Yeast Nucleic Acid

Experiment	Per cent original cytidine-uridine recovered after*		Per cent inorganic phosphate liberated after	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.
Cytidine				
0.1 N sulfuric acid		100		
0.2 " " "		105		
1.0 " " "	94	105		
5.5 " " "	81	84		
1.0 " hydrochloric acid	109			
6.0 " " "	50			
Uridine				
0.2 N sulfuric acid		95		
0.4 " " "		100		
Cytidylic acid				
0.1 N hydrochloric acid	75	106	68	94
0.5 " " "	85		90	100
Yeast nucleic acid				
0.4 N sulfuric acid	29	31	94	100

* In the case of cytidylic acid and yeast nucleic acid the amount of mold growth was calculated as pyrimidine nucleoside (9). The cytidine-uridine value in the case of yeast nucleic acid is given as the percentage found in the original sample. The 48 hour value expressed as moles of pyrimidine nucleoside per mole of phosphorus was 0.465.

The results expressed as per cent recoveries of pyrimidine nucleoside and as the per cent of the original cytidylic acid or nucleic acid phosphate hydrolyzed are summarized in Table I. It may be seen that cytidine and uridine activity for the mold was not affected when either cytidine or uridine was refluxed for 24 or 48 hours in concentrations of acid that wer 1 N or less. Some loss of pyrimidine nucleoside activity took place in 5. N sulfuric acid after 24 hours, and in 6 N hydrochloric acid after the sam length of time, the amount corresponded to 50 per cent of the cytidin originally present. Cytidylic acid was not completely hydrolyzed to cyti dine or uridine and inorganic phosphate after 24 hours in either 0.1 N or 0. N hydrochloric acid. After 48 hours, however, hydrolysis was eithe complete or practically so for the two acid concentrations as measure by both inorganic phosphate formed and pyrimidine nucleoside activit recovered.

The results with yeast nucleic acid were comparable to those with cyti dylic acid. A value of 94 per cent of the original nucleic acid phosphoru was present as inorganic phosphate after 24 hours, as compared to 100 pe cent after 48 hours. The molar pyrimidine nucleoside to phosphoru ratio reached a maximum of 0.465 after 48 hours and decreased to a valu of 0.45 after an additional 24 hours of refluxing. The difference betwee these values, while possibly of questionable significance in terms of th precision of the assay procedure, showed a slight destruction of pyrimidin nucleosides after the 72 hour period of heating.

Conversion of Cytidine to Uridine—Proof that cytidine was largely con verted to uridine during a 48 hour hydrolysis with 0.4 N sulfuric acid wa provided in four ways; namely, (*a*) by assaying portions of the hydrolysi mixture for uridine after phospho-12-tungstic acid precipitation, (*b*) b spectrophotometric analysis for cytidine and uridine during the hydrolysis (*c*) by estimation of the ammonia formed, and (*d*) by isolation of crystal line uridine from the hydrolysis mixture.

Assay of Hydrolysis Mixture for Uridine—A solution containing 100 mg of cytidine sulfate in 10 ml. of 0.4 N sulfuric acid was refluxed over a pe riod of 48 hours. A 1 ml. aliquot was removed for the zero time control an successive 1 ml. aliquots were removed at the end of 1, 2, 4, 8, 16, 32, an 48 hours. The samples were diluted to 2 ml., 1 ml. of this solution wa diluted to 10 ml., and three 1 ml. aliquots were used for assay of total py rimidine nucleosides with the *Neurospora* mutant 1298. The remaining ml. of the original diluted 1:2 was transferred to a 15 ml. centrifuge tub and diluted to about 5 ml. with water. 3 drops of concentrated hydro chloric acid were added, the solution heated in a boiling water bath, 0. ml. of phosphotungstic acid solution (400 mg. per ml. in N hydrochlori acid) added, and the tubes placed in an ice bath in the refrigerator over night. Another 0.1 ml. of phosphotungstic acid was added to insure a excess and the tubes again set in the refrigerator overnight. The precipi tate was centrifuged and the supernatant decanted; the precipitate wa washed with 1 ml. of cold N hydrochloric acid, and the washings added t the supernatant. 1 ml. of ammonium chloride solution was added to the combined solutions, which had been heated in a water bath, and the suspension allowed to stand in the cold overnight. The precipitate was centrifuged and washed with 1 ml. of N hydrochloric acid; the combined supernatants were diluted to 25 ml., and 2 ml. samples in triplicate were used for uridine assay with the mold.

The average mycelium weights and the corresponding values for total pyrimidine nucleosides and for uridine, determined from the growth curve (9) and expressed as concentration in the original hydrolysis mixture, are recorded in Table II. Although the uridine results after the shorter periods of hydrolysis cannot be considered very accurate because of the small amounts of mycelium obtained, it may be seen that after 48 hours approximately 70 per cent of the cytidine had been converted to uridine, as determined by this method.

Table II

Conversion of Cytidine to Uridine As Determined by Uridine Assay Procedure with Neurospora Mutant 1298

Time	Total pyrimidine nucleoside		Uridine		Per cent deamination
	Dry weight mycelium	Concentration in original solution	Dry weight mycelium	Concentration* in original solution	
hrs.	*mg.*	*mg. per ml.*	*mg.*	*mg. per ml.*	
0	21.6	7.6	0.7	−0.20	
1	21.5	7.6	2.3	+0.04	0.53
2	21.1	7.4	1.9	0.02	0.27
4	20.8	7.2	2.9	0.30	4.16
8	21.8	7.7	4.6	0.80	10.4
16	21.1	7.4	8.2	2.30	31.1
32†	22.3	7.8	12.4	4.30	55.2
48†	24.4	8.6	16.3	5.80	67.5

* Corrected for the small amount of cytidine present due to the slight solubility of cytidine phosphotungstate (9).

† The relatively high values for total pyrimidine nucleoside found after 32 and 48 hours are probably explained by concentration of the solution during refluxing. The per cent deamination in all cases is calculated on the basis of the total pyrimidine nucleoside found before treatment with phosphotungstic acid.

Spectrophotometric Analysis of Hydrolysis Mixture—The absorption curves of cytidine and uridine in phosphate buffer at pH 7.0 over the wavelengths from 220 to 300 mμ are given in Fig. 1 (11). It is evident that the absorption maxima for the two nucleosides are sufficiently different to distinguish between the two in pure solutions and that relatively large changes in molecular extinction occur, particularly at 230 and 260 mμ, as cytidine is converted to uridine. In a solution in which both compounds are present the total absorption at a particular wave-length or, as can be determined in the Beckman spectrophotometer, the optical density, D, is the sum of th densities due to cytidine and to uridine. If the molecular extinctio coefficients are known for each compound at two wave-lengths and th optical density is determined, simultaneous equations can be written from the Beer-Lambert law in which the concentrations of cytidine and uri

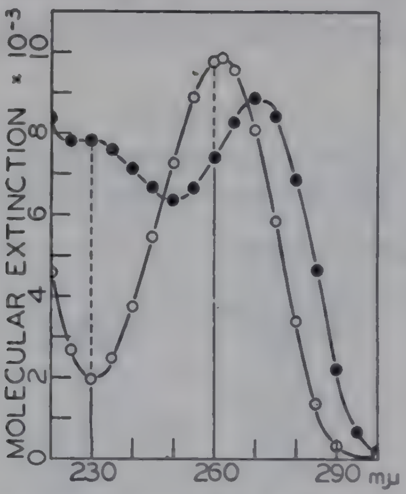

FIG. 1. Absorption curves of cytidine, ●, and uridine, ○, in 0.05 M potassium phosphate buffer at pH 7.0.

dine are expressed as a function of the molecular extinction coefficients and the optical densities at the respective wave-lengths, as follows:

$$C_{u.}E_{u.\lambda_1} + C_{c.}E_{c.\lambda_1} = D_{\lambda_1}$$

and

$$C_{u.}E_{u.\lambda_2} + C_{c.}E_{c.\lambda_2} = D_{\lambda_2},$$

where $C_{u.}$ and $C_{c.}$ are the respective concentrations of uridine and cytidine expressed as mole per liter, $E_{u.\lambda_1}$, $E_{c.\lambda_1}$, $E_{u.\lambda_2}$, and $E_{c.\lambda_2}$ are the respective molecular extinction coefficients of uridine and cytidine at λ_1 and λ_2, and D_{λ_1} and D_{λ_2} are the optical densities at the two wave-lengths Solution of the two equations for $C_{u.}$ and $C_{c.}$ by use of molecular extinction coefficients for uridine and cytidine of 1960 and 7810, respectively, at 230 mμ, and of 9750 and 7390, respectively, at 260 mμ, give the following:

$$C_{u.} = \frac{7810D_{260} - 7390D_{230}}{61.7 \times 10^6}$$

and

$$C_{c.} = \frac{9750D_{230} - 1960D_{260}}{61.7 \times 10^6}$$

where D_{230} and D_{260} are the optical density measurements at 230 and 260 mμ, respectively.

The conditions employed in following the conversion of cytidine to uridine spectrophotometrically were similar to those employed in the first experiment. In this case the solution contained 700 mg. of cytidine in 50 ml. of 0.4 N sulfuric acid. It was refluxed for a total of 48 hours and 0.05 ml. samples were removed after the same intervals mentioned previously and diluted to 25 ml. with 0.05 M potassium phosphate buffer at pH 7.0. Density measurements were made at 230 and 260 mμ, and the respective concentrations of cytidine and uridine in the original mixture calculated by means of the equations given above. The respective values are given in Table III, together with the percentage deamination with time. It may be seen that the rate of deamination found in this experiment was in good agreement with that measured by the *Neurospora* assay procedure at the higher concentrations of uridine.

TABLE III

Conversion of Cytidine to Uridine As Determined by Spectrophotometric Analyses for Uridine and Cytidine during Refluxing for 48 Hours with 0.4 N Sulfuric Acid

Time	Concentration found × 10²*		Total uridine + cytidine found × 10²	Per cent deamination
	Uridine	Cytidine		
hrs.	*moles per l.*	*moles per l.*	*moles per l.*	
0	0.105	6.69	6.79	1.6
2	0.284	6.68	6.96	4.1
4	0.568	5.56	6.14	9.3
8	1.08	5.00	6.08	17.8
16	1.99	4.56	6.55	30.3
32	3.05	3.03	6.08	50.1
48	4.09	2.24	6.33	64.6

* All concentrations refer to the original reaction mixture.

The data obtained spectrophotometrically were plotted in Fig. 2 as the log $1/F$ against the time in hours, where F is the fraction of cytidine remaining at the respective times. It may be seen from Fig. 2 that a straight line typical of a first order reaction was obtained. The velocity constant was calculated and found to be 0.023 per hour.

Ammonia Determination—The ammonia present after 48 hours in the above-described hydrolysis mixture was determined by nesslerization, according to the method of Johnson (12). The amount found calculated as moles per liter was 3.94×10^{-2}, a value comparing favorably with the final uridine concentration of 4.09×10^{-2} mole per liter found spectrophotometrically.

Isolation of Uridine—The remainder of the hydrolysis mixture was evaporated to 10 ml. under an air stream and concentrated phosphotungsti acid solution was added until no further precipitation occurred. Th mixture was left in an ice bath in the refrigerator overnight. The precipi tate was centrifuged, washed three times with 1 ml. portions of N sulfuri acid, and barium hydroxide added to the supernatant liquid and washing until no more precipitation occurred. The mixture was filtered (Celite and the filter cake washed twice with hot water. The resulting solutio was freed of barium ions by treatment with sulfuric acid and the final fil trate was evaporated to a small volume under an air stream at about 30° and then to dryness *in vacuo* over phosphorus pentoxide. The residue wa suspended in hot absolute alcohol and dissolved by the addition of 20 drop of water. On cooling the solution the uridine crystallized, giving a yield o 154 mg. of product with a melting point of 160–165°. The theoretical yiel

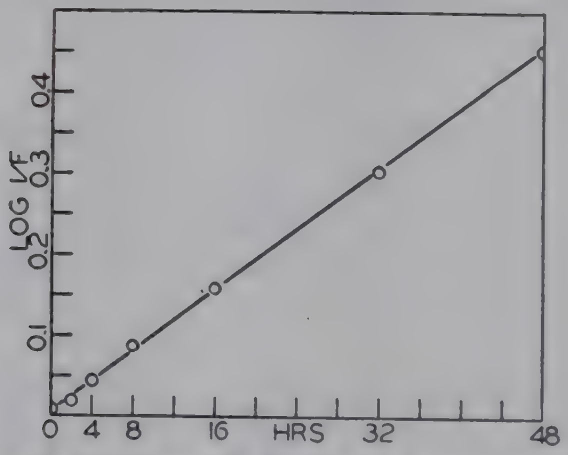

FIG. 2. Deamination of cytidine. *F*, fraction of cytidine present at respective times.

calculated from the spectrophotometric data was 498 mg. The product was dissolved in 5 ml. of water and the solution, after decolorizing by boiling with 0.2 gm. of norit, was filtered and evaporated to dryness at room temperature. The syrup was dried *in vacuo* over phosphorus pentoxide. 5 ml. of hot absolute alcohol were added and water dropwise, until the solid was completely in solution. Crystallization occurred as soon as the solution was cooled, and the beaker and solution were rubbed with a glass rod. The dry product melted at 163–165° in agreement with the reported value for uridine ((2) p. 170).

Preparation of Uridine from Yeast Nucleic Acid—25 gm. of yeast nucleic acid (Schwarz HN-4625) were added to 250 ml. of 0.4 N sulfuric acid and refluxed for 2 hours. Enough finely powdered silver sulfate (14 gm.) was added to provide a slight excess of silver ions (tested with dilute hydrochloric acid). The mixture was placed in the cold room in an ice bath overnight and was filtered with the aid of Celite. The silver purines were washed with two 25 ml. portions of cold 0.4 N sulfuric acid and the washings combined with the filtrate. The excess silver ion was removed from the filtrate with 1 N hydrochloric acid (about 12 ml. required). The silver chloride was filtered hot, and the resulting solution refluxed for 46 hours. A solution containing 15 gm. of phosphotungstic acid was added to the hot solution and the mixture placed in the cold room in an ice bath overnight. The insoluble phosphotungstates were filtered in the cold with the aid of Celite. The filter cake was washed twice with water. The washings were combined with the filtrate, which was heated, and enough crystalline barium hydroxide added to bring the solution to about pH 8 (green with Fisher Alkacid paper). About 40 gm. of barium hydroxide were required. The insoluble barium phosphate, sulfate, and phosphotungstate were removed from the hot solution by filtration and the filter cake was washed twice with boiling water.

35 ml. (wet volume) of a cation exchanger Duolite C-3[2] (the commercial product was washed with acid and alkali and with water four times before it was used) were added and the mixture shaken about 4 hours. The resulting pH was 3. The Duolite was filtered and washed two times with water, and the washings combined with the filtrate. 15 gm. of dry anion exchanger Duolite A-2 (36 ml. wet volume) were exhausted and regenerated twice, and washed until the washings were neutral to litmus. The eluate from the Duolite C-3 treatment was added and the mixture stirred for 2 hours. The pH went up to 6 and the solution became colorless. The ultraviolet absorption of an aliquot at 260 mμ indicated that a total of 3.8 gm. of uridine was present. The solution was evaporated to 100 ml. *in vacuo* at about 50° and then blown off with warm air to dryness. The syrup was dried *in vacuo* over phosphorus pentoxide. The resulting glass was heated with 100 ml. of absolute alcohol and enough water was added slowly to the hot solution to dissolve the precipitate. The solution was seeded with uridine and cooled in the refrigerator. Crystallization began immediately. The crystals were filtered and dried over phosphorus pentoxide, 2.25 gm. of product with a melting point of 164–165° being obtained. A second yield of 1.02 gm. which melted at 161–163° was obtained from the mother liquors, giving a total of 3.27 gm. or 13.1 per cent based on the original nucleic acid. In another preparation in which 100 gm. of yeast nucleic acid were used, the yield was 12.3 per cent. The twice recrystallized product melted at 166–167°. It contained 11.51 per cent nitrogen by the micro-Dumas method,[3] as compared to the theoretical of 11.47 for $C_9H_{10}O_6N_2$.

Preparation of Cytidine from Yeast Nucleic Acid after Acid Hydrolysis—100 gm. of yeast nucleic acid (Lemke) were added to 1 liter of 0.4 N sulfuric acid and the mixture refluxed for 14 hours. The hot solution was made slightly alkaline to phenolphthalein paper with crystalline barium hydroxide (about 160 gm.) and freed from barium sulfate and barium phosphate by filtration. The precipitate was washed twice by suspending in boiling water and filtering, and the washings and the original filtrate were treated with a solution containing 25 gm. of silver sulfate in water with enough concentrated ammonia added to dissolve the precipitated silver oxide. The suspension was allowed to stand in the cold room overnight. The precipitate of silver purines was filtered in the cold with the aid of Celite, and the filter cake was washed twice with cold water. The filtrate and washings were made acid to litmus with sulfuric acid and the excess silver precipitated with hydrogen sulfide. The filtrate was made alkaline to phenolphthalein with barium hydroxide and concentrated *in vacuo* to a small volume. 5 ml. of concentrated nitric acid and about 50 ml. of alcohol were added to the cold solution. Crystals of cytidine nitrate separated when the beaker was rubbed with a glass rod. After 3 hours in the cold, the crystals were filtered, washed with alcohol, and air-dried. The yield was 3.1 gm. After decolorizing with norit and recrystallization, the product melted, with decomposition, at 197°.

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[2] Kindly provided by the Chemical Process Company, San Francisco, California.

[3] Micro-Dumas nitrogen analyses by the Laboratory of Microchemistry, New York.

Preparation of Free Cytidine by Anion Exchange—2 ml. of Duolite A-2 were washed, exhausted with 1 N hydrochloric acid, and regenerated with 4 per cent sodium carbonate. The resin was recycled again and finally washed eight times with distilled water and placed in a 10 mm. column. 1 gm. of cytidine nitrate was dissolved in 10 ml. of warm water and passed through the column at a rate of approximately 20 volumes per hour. The resin was washed out with three 3 ml. portions of warm water. The aqueous solution of free cytidine was concentrated to a syrup under an air stream at room temperature and the remaining water was removed in a vacuum desiccator over phosphorus pentoxide. The resulting glass was warmed gently with 10 ml. of absolute alcohol, 20 drops of 1:10 ammonia were added, and the hot solution seeded with cytidine. Crystals began forming immediately. The solution was cooled and then filtered. The product was dried in a vacuum desiccator. Yield, 746 mg., or 94 per cent of the theoretical. Melting point, 213–214°. This value is lower than that reported by Levene ((2) p. 169), but another authentic sample of free cytidine gave the same melting point. Recrystallization from alcohol-water gave a product melting at 215–216°. The nitrogen content found by the micro-Dumas procedure was 17.21 per cent, as compared to the theoretical of 17.28 per cent for $C_9H_{11}O_5N_3$.

SUMMARY

The stability of cytidine and uridine to acid hydrolysis has been determined in comparison with the rates of liberation of inorganic phosphate

from cytidylic acid and from yeast nucleic acid. When cytidine was heated with 6 N hydrochloric acid for 24 hours, it was largely destroyed as measured by the recovery of pyrimidine nucleoside activity for the *Neurospora* mutant 1298. In 0.1 N, 0.2 N, 0.4 N, and 1 N acid, however, nearly quantitative recoveries were found with cytidine and uridine after 24 or 48 hours. After refluxing cytidine for 48 hours with 0.4 N sulfuric acid, fractionation of the resulting solution showed that the aminopyrimidine was largely converted to uridine and ammonia under these conditions. As both cytidylic acid and yeast nucleic acid are completely hydrolyzed to inorganic phosphate under the same conditions, this procedure has provided a simplified method for the preparation of uridine. Yields from 12.3 to 13.1 per cent of the original nucleic acid were obtained. After refluxing yeast nucleic acid with 0.4 N sulfuric acid for 14 hours, cytidine nitrate was isolated in a yield of 3.1 per cent.

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ON THE NATURE OF LABILE PROTEIN

I. THE CATHEPSIN II ACTIVITY OF THE LIVER AND KIDNEY OF THE FED AND FASTED RABBIT*

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When rats are fasted 24 to 48 hours, as much as 20 to 30 per cent of the hepatic protein leaves the liver (1, 2). As the fast continues to 7 days, this figure reaches 40 per cent (3). In terms of metabolism, the protein, which readily leaves the liver, has been called labile protein or storage protein (1), while the more stable protein has been looked upon as structural protein of the cell. It was pointed out in 1936 that from a chemical point of view no protein fraction of the liver can be regarded as reserve material in the sense of being chemically distinct from the basal structural proteins (4). Various hypotheses have been offered to explain this phenomenon (4–6).

More recently Kosterlitz (7) developed the concept of "labile liver cytoplasm" in which the labile protein is part of a phospholipide-ribonucleic acid complex making up the chromophilic granules of the cytoplasm (8). Here again any claim that there is a "chemical distinction between the labile and the remaining liver cytoplasm" is cautiously excluded from the concept. It was further suggested, on the basis of the reports of Lazarow (9) and Schmitt (10), that enzyme activity may be associated with the cytoplasmic particulates. Soon thereafter Schneider found that the non-nuclear fragments of hepatic homogenates contained the greater proportion of the succinoxidase, cytochrome oxidase, and ATPase activity of the cell (11). Brachet and Jener found catheptic activity in the cytoplasmic particulates studied by them (12).

Finally within the past year Miller (13) reported that the loss of protein from the liver was related to the loss of enzyme activity. He proposed that the enzymes of the cellular particulates represent functional units, the loss of any portion of which is associated with the loss of the unit as a whole.

From these observations the present author believes that labile protein may be chemically distinct from the more metabolically stable protein of the cell. This distinction is based on additional evidence to be presented here, which suggests that the enzymatic distinction of labile protein is

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indicative of a chemical difference in the protein moiety of the "functio unit" or the labile cytoplasmic particulates. This difference depends the extent to which the specificity of an enzyme is determined by its chemi composition and configuration.

The term "labile enzyme" is suggested as a term to aid in the charact ization of enzyme systems supported by labile protein.

In the first of a series of proteolytic enzymes to be examined on this ba we have selected cathepsin II, a component of the endocellular enzy cathepsin, shown by Fruton *et al.* to be multiple in nature (14).

In the present studies, the kidney, whose protein is more stable me bolically than that of the liver, was also analyzed to determine to what gree the observations made are peculiar to the liver. The significance these data in relation to the rôle of cathepsin II in protein synthesis also be discussed.

Procedure

Thirteen female rabbits weighing from 3 to 4 kilos were used in th experiments. Five were fasted for 6 days and eight were allowed to the stock diet of Purina chow *ad libitum*.

The animals were exsanguinated from the carotid. The organs w removed immediately, weighed, and frozen intact in a dry ice cham Homogenates were prepared from each tissue as follows: Both kidneys 15 to 20 gm. of liver) were chipped in the frozen state and placed toget with 10 times their weight of ice water containing chipped ice in a w chilled metal Waring blendor. Under these circumstances the blen remained ice-cold during the 1 to 2 minute period of homogenization. T suspension was then removed and brought to volume. Aliquots of th homogenates were used for semi-micro-Kjeldahl and enzyme stud

Method

Although a variety of techniques has been described by other investi tors for the determination of the cathepsin II activity of tissues (15–1 none have utilized total organ homogenates as the test medium. For t reason sufficient data were accumulated to show that under conditions these experiments the first order reaction constant for the liberation ammonia from benzoylargininamide is proportional to the amount of hom genate used and therefore the enzyme concentration. Fig. 1 shows results obtained on three livers of fed rabbits and three livers of fas rabbits.

In carrying out the tests 1.0 ml. of homogenate was added to 1.0 solution of 0.1 M citrate buffer containing 0.02 mM of benzoylargininam hydrochloride monohydrate, and 0.01 mM of cysteine hydrochloride. T

H of the buffer was 5.1. When less than 1.0 ml. of homogenate was used, he total volume of digest was brought to 2.0 ml. with water. The digesion was carried out in large test-tubes placed in a 39° bath. After 2 and 4 ours of digestion, the tubes were removed, 1 ml. of saturated potassium arbonate and 3 ml. of water were added, and the whole aerated as in the Van Slyke urea aeration procedure described by Greenstein and Leuthardt (17). In the present investigations the ammonia liberated was absorbed n a 2 per cent boric acid solution containing indicator (methyl red-bromcresol green). The ammonia was titrated with N/14 HCl.

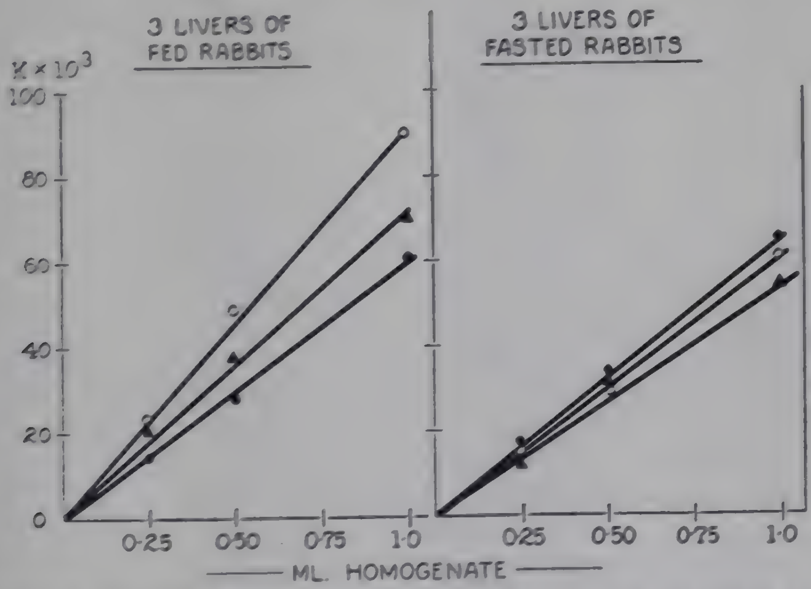

FIG. 1. To each aliquot of total homogenate of liver, as indicated in Fig. 1, there were added 0.02 mM of benzoylargininamide, 0.01 mM of cysteine hydrochloride in 0.2 M citrate buffer (pH 5.1), and the whole was brought to 2.0 ml. with water. K was calculated from the NH_3 liberated by the equation $K = 1/t \log (100/100 - x)$, where t is in hours and x = per cent hydrolysis.

A correction blank for zero time was made in tubes that were prepared, but treated immediately with saturated K_2CO_3. In each case the reaction constant was calculated from the equation $K = 1/t \log (100/100 - x)$, where x was per cent hydrolysis and t was taken in hours. For routine work where a large number of analyses were made, it was found convenient for the numerous calculations required to use t in hours. The expression K per mg. of N was calculated by dividing the K value of 1 ml. of homogenate by the mg. of N found in 1 ml. of homogenate. The expression K per mg. of N was retained here rather than the proteolytic coefficient C, used by others (14), in order not to cause confusion, for the conditions are not exactly the same. By multiplying the K value for 1 ml. by the total volume of homogenate a value for the total enzyme activity of the organ was obtained; this is referred to as "total K" in Table I.

Results

The results in Table I show that in the course of a 6 day fast there was approximately a 50 per cent loss of liver weight and liver nitrogen along with an average body weight loss of about 10 per cent. In the kidneys the

TABLE I

Cathepsin II Content and Concentration of Organs of Fed and Fasted Rabbits

Rabbit No.	Body weight		Organ weight		Nitrogen		Enzyme activity*		
	Initial	Final		Of body weight	Of organ	Of total organ	K per mg. N $\times 10^3$	K per gm. tissue $\times 10^3$	Total K tissue
	gm.	*gm.*	*gm.*	*per cent*	*per cent*	*mg.*			
			Livers fasted						
19	4590	3960	80.7	2.04	2.29	1845	22	517	46.0
20	3987	3807	62.5	1.64	2.63	1645	17	440	27.5
22	4117	3910	67.2	1.37	2.84	1045	24	505	33.9
23	3814	3480	48.7	1.40	2.68	1305	16	425	20.7
25	3627	3274	49.9	1.52	2.95	1470	15	680	22.2
Average			62.0			1460	19	513	30.0
S.E.			6.0			40	2	45	4.0
			Livers fed						
35		3500	131.0	3.76	2.08	2730	28	585	76.5
36		3775	135.0	3.58	2.14	2900	30	640	86.5
37		2975	100.0	3.35	2.29	2290	27	625	62.4
38		3175	98.2	3.08	2.13	2090	45	955	93.5
47		4080	159.6	3.91	2.84	4530	28	810	129.0
48		3580	111.1	3.11	2.24	2495	38	805	95.0
49		4186	143.0	3.42	2.68	3830	33	885	127.0
50		4135	94.0	2.29	2.76	2610	31	863	82.0
Average			119.0			2934	32	784	94.0
S.E.			8.0			290	2	50	8.0
			Kidneys fasted						
19			15.8	0.40	2.46	389	26	638	10.1
20			13.3	0.35	2.87	330	14	401	5.3
22			16.3	0.32	2.36	348	10	216	3.5
23			12.1	0.41	2.77	336	11	314	3.8
25			11.9	0.35	2.96	292	8	196	2.3
Average			13.9			346	14	353	5.0
S.E.			1.0			16	2	80	1.3

TABLE I—*Concluded*

Rabbit No.	Body weight		Organ weight		Nitrogen		Enzyme activity*		
	Initial	Final		Of body weight	Of organ	Of total organ	K per mg. N $\times 10^3$	K per gm. tissue $\times 10^3$	Total K tissue
	gm.	*gm.*	*gm.*	*per cent*	*per cent*	*mg.*			
				Kidneys fed					
35			25.6	0.73	1.75	475	25	437	11.2
36			20.8	0.554	2.29	475	27	615	12.8
37			17.1	0.575	2.36	403	28	658	11.4
38			15.8	0.498	2.51	398	26	667	10.5
47			19.5	0.476	2.62	510	23	602	11.7
48			14.8	0.414	2.67	395	42	1120	16.6
49			19.3	0.461	2.69	520	22	582	11.2
50			16.2	0.391	2.56	415	31	924	12.8
Average			18.5			449	28	701	12.3
S.E.			0.9			22	2	76	0.7

* Enzyme activity here is calculated in terms of the first order reaction constant K from the equation $K = 1/t \log (100/100 - x)$, where t is in hours and x in per cent hydrolysis of benzoylargininamide determined as described in the text. Total K is calculated from the total volume of organ homogenate and the K value of the aliquot used in the test. S.E. is the standard error of the mean.

loss of weight was 25 per cent and the renal N was diminished by 20 per cent.

Two-thirds of the enzymatic activity of the liver and more than half of that of the kidney were lost during the 6 day fast. Thus the enzymatic activity decreased to a greater extent than the liver weight or kidney weight. This is reflected in the lowered ratio of K per mg. of N and K per gm. of tissue in the organs of the fasted rabbits.

DISCUSSION

It has been suggested that cathepsin II may be the endocellular enzyme whose physiological function is concerned with protein synthesis (14, 19). These data may offer further support to this hypothesis. For, since the value of K per mg. of N in Table I is a measure of the proportion of enzyme to protein, it is obvious that in a state of decreased protein synthesis the ratio of enzyme activity to protein was diminished. This is to be expected if cathepsin II were involved in the synthesis of protein. This is a case in which the activity of cathepsin II does not parallel that of cathepsin, if one is permitted to compare the rat and the rabbit (13).

It is accepted here as a working hypothesis that the loss of enzyme activity is a reflection of the loss of labile protein. Support for this can be found in the work of Miller (13) and in Woolley's recent report (20) that strepogenin contains a polypeptide common to biologically active factors which include more than one enzyme. It is obvious that, if the labile protein contains units common to more than one enzyme, the activity of this group of enzymes will be altered. As a first approximation of the extent to which the activity of an enzyme depends on the labile protein the *K* per mg. of N value of the labile protein was calculated by dividing the loss of enzyme activity by the loss of labile protein. The "enzyme activity" of labile protein for cathepsin II has been compared with values calculated from data in the literature.

The labile protein is richer in factors supporting cathepsin II activity than is the cellular protein of the fed or fasted liver. This can readily be seen in Table I, where the labile protein, the protein N lost on fasting, is 1.5 gm., while the loss of enzyme activity is 60; the *K* per mg. of N of the labile protein is therefore 40×10^{-3}, which is greater than the average of 32×10^{-3} found for the livers of the fed animals and twice as great as the 19 ± 2 found for the more stable protein remaining in the cell.

A similar analysis of the data of Miller offers additional information on the nature of labile protein. In the livers of female rats catalase, alkaline phosphatase, cathepsin, and xanthine oxidase showed a labile enzyme-labile protein ratio (in units per gm. of protein) of 16, 6.5, 99, and 23, respectively, while the ratio of the 7 day fasted livers were 5.4, 5.7, 110, and 4.9 respectively.

In the male rats, the values for labile enzymes per gm. of labile protein were (in the same order) 18.3, 5.3, 66, and 26. The figures for the livers of the 7 day fasted rats of the same series contained 12.6, 6.4, 92, and 4.9 enzyme units per gm. of hepatic protein. Thus it can readily be seen that the labile protein supported more than twice the activity of catalase and more than 4 times the activity of xanthine oxidase than did the protein remaining after a 7 day fast. In the male rats the value for catalase is 50 per cent greater but for xanthine oxidase it is of the same order of magnitude as in the female. Cathepsin activity appears to depend on the labile protein to a lesser degree than on the more stable protein. Although there is a 100 per cent difference in the catalase activity of the livers of the fed female and male rats, the values of *K* per gm. of N of the labile protein in both cases are remarkably alike, being 16, 400, and 18,300 respectively.

From the data of Oppenheimer and Flock (21) on fed and 48 hour-fasted rats, the labile protein was calculated from the loss of tissue weight by using a conservative value of 20 per cent loss of N (1) and 3.0 per cent for the N content of the tissue. The *K* per mg. of N value of the labile protein for alkaline phosphatase was 4.4 Bodansky units per 100 mg. of N. The value of the protein remaining in the liver was 0.2 Bodansky unit per 100 mg. of N.

On the basis of the findings reported here and by Miller and Flock and Oppenheimer, one can point to catalase and xanthine oxidase in the 7 day-fasted rat, to phosphatase in the 48 hour-fasted rat, and to cathepsin II in the 6 day-fasted rabbit as representing a group of labile enzymes. Their activity depends more on the constituents of the metabolic pool of hepatic protein which readily leaves the liver on fasting than on the metabolically stable protein remaining in the cell. To the extent that this protein supports their activity, the nature of these enzymes may be a manifestation of chemical properties that are peculiar to labile protein and distinct from the more stable protein of the cell. Further studies on the significance of the labile protein in relation to the labile enzymes and their rôle in protein synthesis in the liver will be the subject of future investigations.

SUMMARY

It has been shown that total homogenates of the liver are suitable for quantitative studies of cathepsin II.

The cathepsin II of the liver and kidneys of eight fed and five fasted rabbits was determined.

The term "labile enzyme" has been used to describe the enzyme activity readily lost on fasting.

A point of view was developed whereby a chemical distinction can be made between the labile protein and the more stable cellular proteins. This view is based on the principle that the cellular proteins supporting enzymatic activity possess chemical composition and configuration specific for the enzyme.

On this basis labile protein is so constituted as to be more capable of supporting the activity of cathepsin II and other enzymes reported by others than the more stable cellular proteins.

Cathepsin II may be related to protein synthesis in that the level of its activity parallels the relative rate of synthesis and catabolism of protein in the liver.

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STUDIES ON PITUITARY LACTOGENIC HORMONE

XIII. THE AMINO ACID COMPOSITION OF THE HORMONE OBTAINED FROM WHOLE SHEEP PITUITARY GLANDS*

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In this paper we wish to present certain analytical data obtained with the lactogenic hormone isolated from sheep pituitaries by the method of Li *et al.* (1). The hormone was shown to be homogeneous by electrophoretic and solubility measurements (2).

EXPERIMENTAL

The lactogenic hormone preparation was dried in a vacuum at 98° for 20 hours before digestion. A weighed amount (approximately 90 mg.) of the dried sample was refluxed with 2 cc. of 20 per cent HCl. At the end of 18 hours, HCl was removed by drying in a vacuum; the residue was dissolved in water and stored in the refrigerator with a trace of toluene and ether pending analysis. The microbiological assay procedures[1] were employed for the determination of arginine, aspartic acid, glycine, histidine, lysine, phenylalanine, proline, serine, tyrosine (with *Leuconostoc mesenteroides* P-60), glutamic acid, isoleucine, leucine, methionine, valine (with *Lactobacillus arabinosus* 17-5), and threonine (with *Streptococcus faecalis*). L-Amino acids were used as standards in all cases (except glycine).

Tryptophan was determined chemically; the procedure of Lugg (3) with alkaline hydrolysates was followed in essential detail. The colorimetric methods of Shaw and McFarlane (4) and of Horn and Jones (5) were also employed for the determination of tryptophan.

The free amino nitrogen was determined in the manometric Van Slyke apparatus (6), and the total nitrogen by the Kjeldahl procedure.

The determination of amide NH_3 was carried out as follows: 50 mg. of protein were hydrolyzed with 20 per cent HCl at 100° for 12 hours; the solution was then neutralized and made slightly alkaline with 0.5 cc. of 5 M NaOH. The ammonia was distilled off by aeration into three aliquots of a borate buffer while the hydrolysate was kept in a boiling water bath.

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* Aided by grants from the Research Board of the University of California, Berkeley.

[1] Lewis, J. C., and coworkers, unpublished data.

DISCUSSION

The amino acid composition of the lactogenic hormone is summarized in Table I. It may be noted that the determinations of arginine and tyrosine by microbiological and chemical methods (3, 9) are in good agreement. The earlier microbiological assay of glutamic acid (8) is not significantly different from the present value. The values of cystine and methionine are taken from an earlier analysis (7).

TABLE I

Amino Acid Analysis of Lactogenic Hormone

Amino acid	Gm. per 100 gm. protein	Methods
Arginine	8.6	Microbiological*
	8.3	Chemical†
Aspartic acid	11.6	Microbiological†
Cystine	3.1	Chemical‡
Glutamic acid	14.1	Microbiological*
	13.4	" §
Glycine	4.0	" *
Histidine	4.5	" *
Isoleucine	7.2	" *
Leucine	12.5	" *
Lysine	5.3	" *
Methionine	3.6	" *
	4.3	Chemical‡
Phenylalanine	4.1	Microbiological*
Proline	6.2	" *
Serine	6.5	" *
Threonine	4.8	" *
Tyrosine	4.7	" *
	4.5	Chemical‖
Tryptophan	1.2	Microbiological¶
	1.2	Chemical‖
Valine	5.9	Microbiological*

* Determined by J. C. Lewis and coworkers.

† The Sakaguchi method was used; see Li *et al.* (2).

‡ The diiodate titration method of Baernstein was used; see Li (7).

§ Taken from Lewis and Olcott (8).

‖ The method of Lugg was used; see Li *et al.* (9).

¶ Determined by M. S. Dunn and coworkers.

There is confusion as to the exact tryptophan content in lactogenic hormone. Some years ago (9) we found the tryptophan content to be 1.19 per cent by the nitrous acid method of Lugg (3); a similar value was obtained by White *et al.* (10) by the procedure of Folin and Marenzi (11).

We (2) also reported a higher value (2.5 per cent) when the glyoxylic acid method (4) was used. These findings are confirmed in the present investigation. In addition, we have obtained a value of 1.27 per cent (see Table II) by the method of Horn and Jones (5). From these results and that of Ross (12) on tobacco mosaic virus, it would seem that the glyoxylic acid method gives values so high that the results obtained may not always be taken as depicting accurately the tryptophan content in proteins. In order to substantiate this contention, we determined the tryptophan content of a number of proteins with the nitrous acid and glyoxylic acid methods and the method of Horn and Jones. The results are summarized in Table II. It is evident that with the exception of globin, pepsin, and lactogenic hormone the tryptophan content obtained by the three methods is in good agreement. It may be recalled that the glyoxylic acid method requires no digestion of the protein. The coloration produced by the reaction of tryptophan residues with the glyoxylic acid may be altered or interfered with by groupings or peptide chains in the protein molecule. This interference may cause either an enhancement or reduction of the chromogenic intensity. In the case of the lactogenic hormone, it would seem that an unknown factor, due to the intrinsic structure of the tryptophan residue, gives rise to a high value when the glyoxylic acid method is employed.

TABLE II

Tryptophan Content of Proteins As Determined by Different Methods*

The results are expressed in per cent of moisture-free protein; each value was the average of at least three determinations.

Protein	Methods		
	Nitrous acid (Lugg (3))	Glyoxylic acid (Shaw and McFarlane (4))	*p*-Dimethylamino-benzaldehyde (Horn and Jones (5))
Human serum albumin	0.43	0.43	0.44
Casein	1.35	1.68	1.37
β-Lactoglobulin	1.97	2.06	1.98
Globin	1.63	2.60	1.58
Edestin	1.50	1.65	1.52
Gliadin	0.79	0.84	0.73
Pepsin	2.73	3.34	2.68
Lactogenic hormone	1.14	2.28	1.27

* We are indebted to the following who furnished samples of proteins: H. Beniam for the samples of human serum albumin and globin, H. S. Olcott for casein and gliadin, D. M. Greenberg for edestin, and E. F. Jansen for β-lactoglobulin. The crystalline pepsin was obtained from the Plaut Research Laboratory.

TABLE III

Minimum Molecular Weight of Lactogenic Hormone

Amino acid	Gm. per 100 gm. protein	Minimum mol. wt.	Assumed No. of residues	Calculated mol. wt.
Cystine	3.1	7,752	4	31,008
Histidine	4.5	3,447	10	34,470
Phenylalanine	4.1	4,029	8	32,232
Tryptophan	1.2	17,016	2	34,032
Tyrosine	4.7	3,855	9	34,695
Mean ± standard deviation				33,287 ± 226

TABLE IV

Composition of Lactogenic Hormone (Molecular Weight 33,300)

Constituent	Gm. per 100 gm. protein	N as per cent of protein N	Estimated No. of residues
Nitrogen	15.86		
Sulfur	1.79		
Amide N	1.0	6.3	24
Arginine	8.6	17.4	17
Aspartic acid	11.6	7.6	28
Cystine	3.1	2.3	4
Glutamic acid	14.1	8.5	32
Glycine	4.0	4.7	18
Histidine	4.5	7.7	10
Isoleucine	7.2	4.8	18
Leucine	12.5	8.4	32
Lysine	5.3	6.4	12
Methionine	3.6	2.1	8
Phenylalanine	4.1	2.2	8
Proline	6.2	4.8	18
Serine	6.5	5.5	21
Threonine	4.8	3.5	13
Tyrosine	4.7	2.3	9
Tryptophan	1.2	1.0	2
Valine	5.9	4.4	17
Total		99.9	291

In order to ascertain the tryptophan content of lactogenic hormone, the microbiological technique[2] was also employed with *Lactobacillus arabinosus* 17-5. It was found that the hormone contains 1.23 per cent tryptophan, which is in agreement with the values obtained by the methods of Lugg and of Horn and Jones. It may, therefore, be assumed that the amount of tryptophan in the lactogenic hormone approximates 1.2 per cent.

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[2] We are indebted to Dr. M. S. Dunn and his colleagues for this determination.

Table III presents calculations of the minimum molecular weight of the hormone, based upon its content in histidine, phenylalanine, cystine, tryptophan, and tyrosine. The value 33,300 is somewhat higher than that obtained by osmotic pressure measurements (2), but it is very close to that determined by ultracentrifugation (10).

In Table IV are shown the protein nitrogen distribution and the estimated number of amino acid residues per mole. The percentage of nitrogen in lactogenic hormone corrected for moisture and ash is found to be 15.86 per cent from an average of many determinations. It may be seen that the known amino acids together with the amide N account for 99.9 per cent of the protein nitrogen.

The free amino nitrogen (Van Slyke) determinations for the hormone gave an average value of 0.74 per cent. When calculated per mole, the estimated number of free amino groups is found to be 18, which is higher than the 12 lysine residues. The difference (18 − 12 = 6) may be taken as indicating the approximate number of free terminal amino groups in the hormone molecule.

SUMMARY

1. The percentages of seventeen amino acids in the lactogenic hormone isolated from whole sheep pituitaries have been determined by microbiological and chemical procedures. 99.9 per cent of the protein nitrogen has been accounted for by its content of seventeen amino acids and the amide nitrogen.

2. The molecular weight has been estimated to be 33,300 by calculations based on the content of five amino acids.

3. Methods for the determination of tryptophan in proteins have been discussed.

I am extremely grateful to Dr. J. C. Lewis and his coworkers for their generosity in carrying out the microbiological assay of this work. The author also wishes to acknowledge the assistance of C. Kalman.

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A FLUOROMETRIC METHOD FOR THE DETERMINATION OF PTEROYLGLUTAMIC ACID*

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(Received for publication, November 23, 1948)

The characteristic fluorescence of the antipernicious anemia factor was suggested as a measure of its concentration in natural materials by Jacobson and Simpson (1) and a quantitative study of the fluorescence of synthetic pteroylglutamic acid (PGA) was made by Villela (2).

Attempts to apply such direct fluorescence measurements to yeast extracts prepared in this laboratory indicated that a variety of interfering substances could greatly augment the intensity of the fluorescent beam. Riboflavin and many of the pterins, for example, are strongly fluorescent under the conditions recommended by Villela. Consequently, a study has been made of the fluorescence of pteroylglutamic acid under controlled conditions and a method has been developed for its quantitative estimation in a variety of synthetic and natural mixtures.

Permanganate oxidation of PGA yields a strongly fluorescent substance, identified as 2-amino-4-hydroxypteridine-6-carboxylic acid (3). In the absence of fluorescent pigments (*e.g.*, xanthopterin) which are attacked by permanganate, the increment in the intensity of fluorescence, on oxidation, is directly proportional to PGA concentration. When interfering pigments are present, the PGA oxidation product can be isolated chromatographically and its fluorescence determined directly.

Method

Apparatus—The intensity of fluorescence was measured in a Farrand microfluorophotometer, with filters recommended by Dr. O. H. Lowry for the microdetermination of thiochrome, xanthopterin, and related substances (4).

Primary filter. Corning No. 5860, transmitting in the ultraviolet at 365 mμ.

Secondary filter. Corning No. 4308 + Wratten gelatin No. 2A + Corning No. 3389, in that order, with Filter 3389 facing the phototube.

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† From a dissertation to be submitted by V. Allfrey in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

The secondary filter combination transmits in the blue region at 470 mμ, blocks out stray ultraviolet radiations, and reduces filter fluorescence to a minimum.

Adsorption columns. Pyrex glass tubes, 5 mm. inside diameter and 120 mm. in length, with a bell-shaped top of 22 mm. inside diameter and 50 mm. in length, and a capillary constriction at the bottom, 1 mm. inside diameter and 15 mm. in length, over-all length 180 mm. When the tubes are charged, the rate of flow should be 15 to 20 drops per minute.

Reagents—Pteroylglutamic acid, crystalline reference standard (generously supplied by Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company), 4 per cent potassium permanganate solution, 3 per cent hydrogen peroxide solution, prepared as needed by 1:10 dilution of 30 per cent H_2O_2, 2 N hydrochloric acid, 4 per cent sodium tetraborate solution ($Na_2B_4O_7 \cdot 10H_2O$), 40 per cent sodium hydroxide solution, and 1 mg. per cent of quinine sulfate in 0.1 N sulfuric acid. Dilutions of this quinine sulfate standard serve as reference points in the selection and adjustment of instrument sensitivity.

2.5 M acetate buffer, pH 3.95, prepared as follows: To 500 ml. of 5 M acetic acid add 105 ml. of 5 N NaOH and dilute to 1 liter.

Florisil, 60 to 100 mesh, prepared as follows: 500 gm. of Florisil are suspended in 2 liters of 4 per cent sodium tetraborate solution and boiled for 30 minutes. After decantation, the process is repeated. The Florisil is washed with distilled water and resuspended in 2 liters of 0.25 M acetate buffer (prepared by 1:10 dilution of the acetate buffer described above). This suspension is boiled for 30 minutes and filtered. The Florisil is washed on the funnel with 10 liters of 0.25 M acetate buffer and air-dried at room temperature.

Procedure

Two procedures are available, depending upon the nature and complexity of the material to be analyzed.

Method I—In the absence of fluorescent pigments which are altered by permanganate oxidation, the following direct method may be applied: To 10.0 ml. of a neutral, unbuffered solution containing 0.01 to 10.0 γ of pteroylglutamic acid per ml. is added 0.1 ml. of 2.5 M acetate buffer. The pH at this point should lie between 3.9 and 4.1. The intensity of fluorescence of the solution is determined, with the appropriate sensitivity setting of the instrument. To the 10.1 ml. of buffered solution is then added 0.05 ml. of 4 per cent potassium permanganate solution, followed after 5 minutes by 0.10 ml. of 3 per cent hydrogen peroxide. The final volume is 10.25 ml. The final pH lies between 4.1 and 4.4. After gentle stirring for 2 to 3 minutes the intensity of fluorescence is again determined.

The increment in fluorescence intensity due to oxidation is directly proportional to the concentration of pteroylglutamic acid. The concentration of PGA may be read directly from a standard linear plot of fluorescence increment (Δ) *versus* concentration.

Standardization—Three such plots have been established. The first (Fig. 1, Curve C) covers the concentration range of 0.01 to 0.1 γ of PGA per ml. The reference point of instrument sensitivity for this range is obtained by setting the galvanometer deflection due to a 0.0024 mg. per cent quinine sulfate solution in 0.1 N sulfuric acid at 70 on the 100 unit galvanometer scale. Points on the curve are obtained as follows: 10.0 ml. of a neutral standard solution containing the indicated concentrations of PGA are adjusted to pH 4.02 by the addition of 0.1 ml. of 2.5 M acetate buffer. The initial intensity of fluorescence is determined and recorded. 0.05 ml. of 4 per cent $KMnO_4$ solution is then added, followed after 5.0 minutes by 0.10 ml. of 3 per cent H_2O_2. After gentle stirring for 2 to 3 minutes the final intensity of fluorescence is determined. The difference, Δ, between initial and final readings is plotted against the concentration of PGA in the original 10.0 ml. of solution.

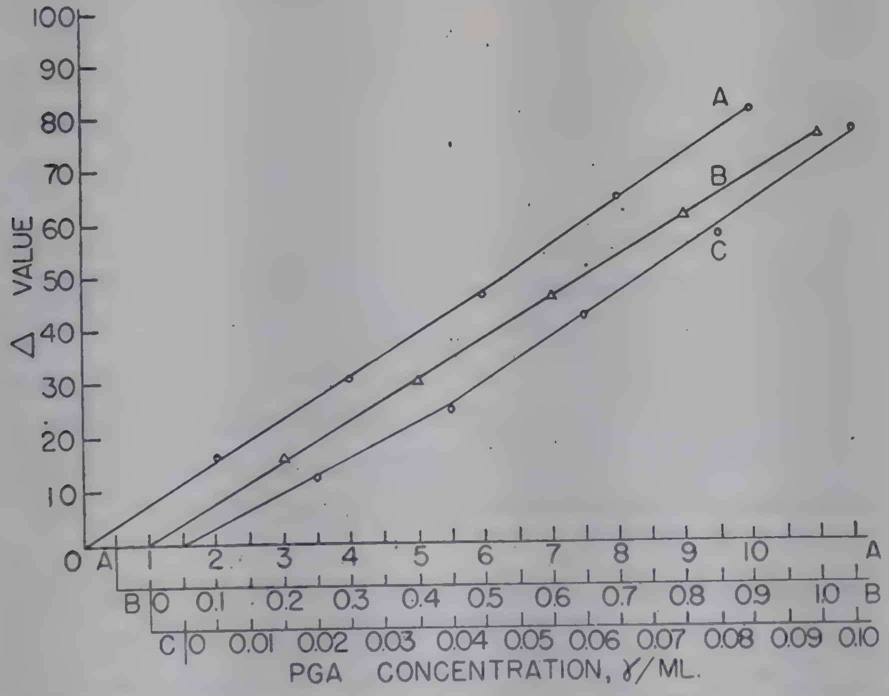

FIG. 1. Fluorescence increment as a function of PGA concentration

As indicated in Fig. 1, Curve C, a slight deviation from linearity occurs at the lower PGA concentrations.

The second standard plot (Fig. 1, Curve B) covers the range from 0.1 to 1.0 γ of PGA per ml. The reference point of instrument sensitivity for this range is obtained by setting the galvanometer deflection due to a 0.024 mg. per cent quinine sulfate solution in 0.1 N H_2SO_4 at 75 on the galvanometer scale. The oxidation technique is the same as that used above.

In this range the increment, Δ, is directly proportional to PGA concentration.

The third curve (Fig. 1, Curve A) applies for concentrations between 1 and 10 γ of PGA per ml. The reference point here is a galvanometer deflection of 75 for a 0.2 mg. per cent solution of quinine sulfate in 0.1 N H_2SO_4.

There is a slight deviation from linearity at the higher PGA concentrations. However, concentrations of this magnitude are not often encountered in the analysis of natural products.

It is of primary importance that the standards used be freshly prepared and shielded from direct sunlight. Exposure to light or long standing results in a partial cleavage of PGA to the corresponding pteridyl aldehyde which fluoresces. The initial intensity of fluorescence of pure PGA standards is no higher than the "blank" fluorescence of the reagents employed.

Method II—When interfering pigments are present, the PGA oxidation product is isolated by adsorption on Florisil at pH 4 and elution in 4 per cent sodium tetraborate solution. The fluorescence of the eluate at pH 4 is determined and recorded. After the addition of 40 per cent NaOH solution the fluorescence is again determined. The difference between initial and final readings (corrected for dilution and the slight fluorescence of the oxidation product in alkaline solution) corresponds to the Δ values of the standard curves in Fig. 1.

The detailed procedure is as follows: To 1 to 10 ml. of sample containing 0.25 to 250 γ of PGA are added 0.1 ml. of 2.5 M acetate buffer and sufficient water to bring the volume to 10.1 ml. The pH at this point should be approximately 4. 0.05 ml. of 4 per cent $KMnO_4$ is then added. (In the presence of high concentrations of citrate, oxalate, or other oxidizable substances, more permanganate may be necessary. Enough permanganate should be present to impart a definite red color to the solution throughout the oxidation period.) After 5 minutes, 0.10 ml. of 3 per cent H_2O_2 is added and the clear solution is passed through about 10 cm. of Florisil in an adsorption column. (Adsorption columns are prepared by inserting a small plug of glass wool above the capillary constriction at the base of the tube and filling to a height of 10 to 11 cm. with dry Florisil. Immediately before using the column is washed with 10 ml. of 0.25 M acetate buffer.) If permanganate causes precipitation in the solution being examined, it is advisable to centrifuge before adsorption. When the sample has passed through the Florisil, the column is washed with five 10 ml. portions of 0.25 M acetate buffer. (Mild suction may be applied to hasten the washing of the column.) To elute, four 5 ml. portions of boiling 4 per cent sodium tetraborate solution are passed through the column. With mild suction an elution rate of 15 to 20 drops per minute can be obtained. The eluate is adjusted to pH 4.0 to 4.5 by the addition of about 1.8 ml. of 2 N HCl, and then diluted to 25 ml. in a volumetric flask.

Duplicate 10 ml. aliquots are withdrawn and 0.1 ml. of 2.5 M acetate buffer is added to each. The intensity of fluorescence is determined on 1 ml. of these solutions before and after the addition of 0.1 ml. of 40 per cent NaOH. Instrument sensitivity is selected by using one of the three quinine sulfate calibration points used for the standard curves. The choice depends on the initial fluorescence of the eluate and the magnitude of the decrease on addition of the alkali.

The difference between the initial reading and the final reading multiplied by the dilution factor 1.1 gives a Δ value which must be further increased by 4 per cent to allow for the fluorescence of the oxidation product in alkaline solution. The corresponding concentration can then be read from the standard curves of Fig. 1.

It is advisable to run "recovery" samples and to correct for losses due to coprecipitation, incomplete elution, or to other causes, depending on the material being analyzed.

In many natural products (*e.g.*, yeast and bacterial extracts) a more complete isolation of the PGA oxidation product is required. The recommended procedure is described in the section covering the application of the method to natural products.

EXPERIMENTAL

Oxidation—As indicated in Table I, the increment, Δ, in fluorescence intensity is essentially constant over a wide range of permanganate concentrations.

10 ml. aliquots of a standard solution containing 2 γ of PGA per ml. were oxidized for 5.0 minutes with 0.05 ml. of the indicated concentrations of potassium permanganate. The solutions were buffered with 0.2 ml. of 2.5 M acetate buffer. Permanganate remaining at the end of the oxidation period was removed with 0.2 ml. of 3 per cent H_2O_2. The difference, Δ, between final and initial intensities of fluorescence was determined at an instrument sensitivity adjustment corresponding to a galvanometer deflection of 34 scale divisions for a 0.024 mg. per cent solution of quinine sulfate in 0.1 N H_2SO_4.

Oxidation Time—The variation of fluorescence increment with time of oxidation is shown in Fig. 2. The increment is constant between 5 and 11 minutes. Data were obtained as follows: 10 ml. aliquots of a standard

PGA solution (1.2 γ per ml.) were buffered with 0.1 ml. of 2.5 M acetate buffer. 0.05 ml. of 4 per cent $KMnO_4$ was then added. After the times indicated by points on the curve, 0.1 ml. of 3 per cent H_2O_2 was added to reduce the remaining permanganate. The increment in intensity of fluorescence due to oxidation was determined at an instrument sensitivity corresponding to a galvanometer deflection of 75 for a 0.024 mg. per cent solution of quinine sulfate in 0.1 N H_2SO_4.

TABLE I

Effect of Permanganate Concentration on Fluorescence Increment

$KMnO_4$ concentration	Fluorescence increment*
per cent	
1	80
2	80
4	80
6	81
8	83
10	83
20	82

* Observed at an instrument sensitivity corresponding to a galvanometer deflection of 34 scale divisions for a 0.024 mg. per cent quinine sulfate standard.

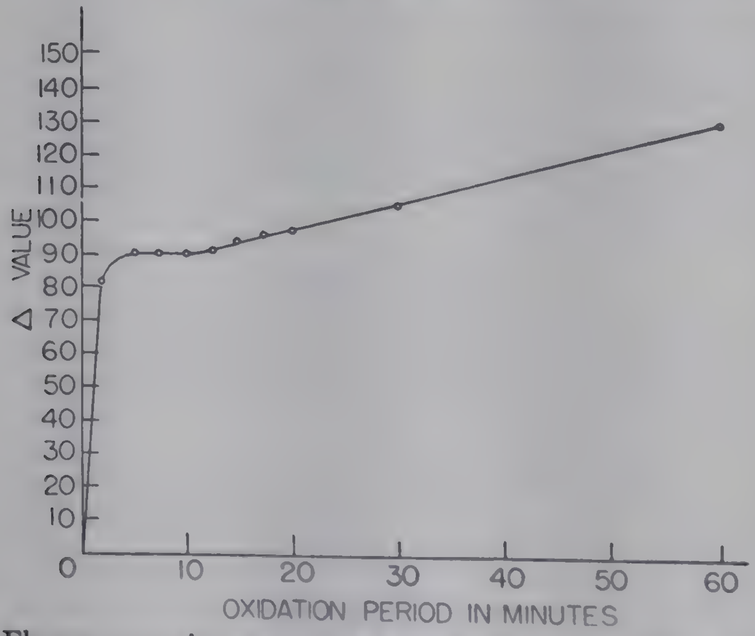

FIG. 2. Fluorescence increment as a function of the time of oxidation

The constancy of the increment between 5 and 11 minutes and its independence, within wide limits, of the permanganate concentration permit a precision in duplicate determinations to within 1 scale division on the galvanometer.

Properties of Oxidation Product—The fluorescence of the oxidation product, as prepared by the adsorption-elution technique outlined above, does not change appreciably within 240 hours. It varies considerably with pH, however, and with the concentration of borate and acetate ions in the solution.

The variation of the fluorescence intensity of the PGA oxidation product with pH is illustrated in Fig. 3. The data were obtained as follows: 12.5 ml. of a PGA standard solution containing 100 γ per ml. were oxidized by the standard procedure and the solution passed through about 10 cm. of Florisil in an adsorption column. The oxidation product was eluted with four 5 ml. portions of boiling 4 per cent sodium tetraborate solution. The eluate was made up to 500 ml. with distilled water. 10 ml. aliquots of this solution were brought to the indicated pH values in a final volume of 25 ml. (pH values below 10 were measured on a Beckman pH meter. Values above pH 10 were calculated from the dilutions of 1.0 N NaOH used in the titrations.) Fluorescence intensities were determined at an instrument sensitivity corresponding to a galvanometer deflection of 75 for a 0.024 mg. per cent quinine sulfate standard.

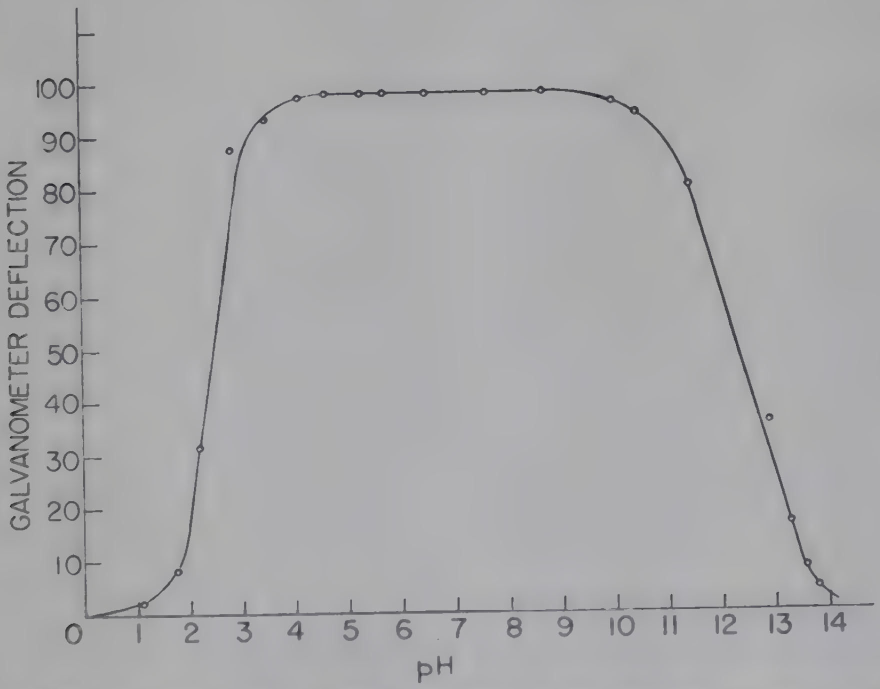

Fig. 3. Fluorescence of the PGA oxidation product as a function of pH

The fluorescence under these conditions is constant between pH 4 and 9.5 and falls off rapidly in more strongly acid and alkaline solutions. The fluorescence intensity of the oxidation product in 1 N NaOH is about 4 per cent of the value determined at pH 4.

In the direct method, in which the oxidation product is not isolated, the constancy of increment holds only between pH 3.9 and 4.4. Above pH 4.4 the formation of MnO_2 causes a significant decrease in the measured increment. The 2.5 M acetate buffer recommended serves to keep the pH within these limits of optimum fluorescence intensity.

Salt Effects—The intensity of fluorescence of the oxidation product varies considerably with its ionic environment. The data in Table II indicate the magnitude of the variation in different concentrations of salts. The final concentration of PGA oxidation product is 1 γ per ml. in all cases. Since the fluorescence of the oxidation product is constant between pH 4 and 9.5, the observed differences probably represent specific ion effects. Fluorescence measurements were made at an instrument sensitivity corresponding to a galvanometer deflection of 42 for a 0.024 mg. per cent quinine sulfate standard. The salt solutions themselves do not give a measurable fluorescence at this sensitivity.

TABLE II

Variation of Fluorescence Intensity of Oxidation Product with Salt Concentration

Solution (containing 1 γ oxidation product per ml.)	Concentration	pH	Fluorescence intensity*
	M		
H_2O		7.1	90
NaCl	0.1	5.1	86
	0.5	5.1	84
	1.0	5.1	80
NaH_2PO_4	0.1	4.45	60
	0.5	4.15	32
	1.0	4.0	22
$(NH_4)_2SO_4$	0.1	5.1	90
	0.5	5.0	97
	1.0	5.0	102
$NaC_2H_3O_2$	0.01	6.85	77
	0.05	7.4	67
	0.10	7.6	65
	0.50	7.7	70
	1.0	7.8	75
$Na_2B_4O_7$	0.01	9.15	83
	0.05	9.15	88
	0.10	9.2	91
	0.15	9.2	94

* Observed at an instrument sensitivity corresponding to a galvanometer deflection of 42 scale divisions for a 0.024 mg. per cent quinine sulfate standard.

An interesting effect is observed in the concentration series for sodium acetate, in which a definite minimum is observed at about 0.1 M. A similar effect is evident in sodium tetraborate solutions in which the initial loss due to "quenching" in 0.01 M solution is restored at 0.1 M (about 4 per cent), and at higher concentrations the fluorescence may exceed that of the water solution.

The extreme "quenching" effects of acetate and phosphate ions emphasize the importance of rigid control in the analytical procedure. Such observations point to the necessity of comparing fluorescence intensities under identical or reasonably identical conditions. It is for this reason that the Florisil eluate is titrated to pH 4 and buffered in acetate before comparison with the standards of Fig. 1.

Adsorption-Elution

General—Barium and calcium carbonates do not adsorb PGA from neutral or weakly alkaline solutions. Both PGA and its oxidation product are partially adsorbed on Decalso and on tricalcium phosphate from neutral or weakly acid solutions. The adsorption of PGA on Magnasol[1] at pH 4 to 5 appears to be quantitative. A number of ion exchange resins were tested for adsorptive capacity, but in all cases these were sufficiently soluble to give excessive and variable "blank" fluorescence readings.

Florisil—Both PGA and its oxidation product are quantitatively adsorbed on Florisil at pH 4. Both are recovered in greater than 90 per cent yield with the recommended elution procedure. Under these conditions riboflavin is adsorbed but not recovered from the column. Xanthopterin is adsorbed and is recovered in good yield. Xanthopterin, however, does not interfere with the method, since permanganate oxidation, prior to adsorption, converts it to leucopterin which does not fluoresce appreciably under these conditions.

The quantitative nature of the adsorption of the PGA oxidation product is illustrated in Columns 2 and 3 of Table III, where the fluorescence intensities before and after adsorption are recorded for different PGA concentration levels. Columns 4 and 6 of Table III list the decrement in eluate fluorescence on addition of 40 per cent NaOH and the corresponding Δ values for PGA standards at the same final concentrations. The data are listed for triplicate determinations. The extent of recovery on elution is recorded in Column 7.

The data were obtained as follows: 10.0 ml. of solution containing the indicated total concentrations of PGA were buffered at pH 4 by the addition of 0.1 ml. of 2.5 M acetate buffer and oxidized by the standard technique previously described. The intensity of fluorescence following oxidation was recorded and the solution was passed through about 12 cm. of Florisil in an adsorption column. The columns were washed with two 10 ml. portions of 0.25 M acetate buffer. The fluorescence intensities of the first filtrates and of the washings were determined and recorded. The columns were then eluted with four 5 ml. portions of boiling 4 per cent sodium tetraborate solution. The eluate was titrated to pH 4 to 4.5 by the addition of 1.7 ml. of 2 N HCl, and diluted to 25 ml. Then 0.25 ml. of 2.5 M acetate buffer was added and the intensity of fluorescence determined on 1 ml. of this solution before and after the addition of 0.1 ml. of 40 per cent NaOH.

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[1] Supplied by the Westvaco Chemical Corporation, New York.

TABLE III

Adsorption-Elution Recovery Data

PGA oxidized and adsorbed	Fluorescence before adsorption	Intensity* after adsorption	Fluorescence decrement of eluate†		Standard Δ value	Recovery
(1)	(2)	(3)	(4)	Average (5)	(6)	(7)
γ						*per cent*
25	86	0	78			
	87	1.5	77	76.3	76.5	100
	86	0	74			
20	68	1	58			
	67	0	58	58	59.5	97
	67	0	58			
15	54	0	45			
	55	0	45	45.7	47.5	96
	56	1.5	47			
10	39	0	36			
	39	0	36	36	35.5	101
	39	0	36			
5	19	0	18			
	19	0	17	17.7	17	104
	19	0	18			
1	39	0	30			
	39	0	31	30	32	94
	39	0	29			
0.5	18	0	10			
	18	0	10	10.7	11	97
	19	0	12			

Fluorescence intensities for 0.5 and 1 γ of PGA oxidized and adsorbed were determined at an instrument sensitivity corresponding to a deflection of 75 scale divisions for a 0.0024 mg. per cent quinine sulfate standard. All other observations were made at a sensitivity of 75 units deflection for a 0.024 mg. per cent quinine sulfate standard.

* Fluorescence intensities corrected for "blank" fluorescence of H_2O controls.

† Decrements corrected for "blank" fluorescence of reagents and fluorescence of the oxidation product in alkaline solution.

The decrease in fluorescence intensity was compared with the Δ values of standard PGA solutions at the same final concentrations and at the same instrument sensitivities.

The data in Table III indicate the essential completeness of the adsorption-elution step. Similar results have been obtained for the isolation of PGA.

The PGA oxidation product may be adsorbed from volumes far in excess of 10 ml., complete recoveries having been obtained for 10 γ of total oxidation product in as much as 200 ml. of solution passing the column. Similarly, washing in acetate buffer at pH 4 can be extended to remove contaminants without a corresponding loss of the oxidation product.

The volume of eluting agent cannot be reduced below 20 ml. without a corresponding decrease in the recovery of oxidation product from the column. Elution at 30° and 60° is likewise incomplete. Two 10 ml. portions may be used in place of four 5 ml. portions of eluting agent without appreciable difference in recoveries. The use of successive small portions at 100°, however, minimizes cooling during elution. Steam-jacketed adsorption columns could be used to advantage at this step.

Interfering Compounds—The direct method (measuring fluorescence increment after oxidation) may be applied without error in the presence of many of the B vitamins, purines, pyrimidines, and amino acids. Riboflavin, for example, in concentrations as high as 10 γ per ml. is unaffected by the oxidation, and PGA is readily determined in admixture with it.

In Table IV are listed many of the compounds which have been examined for their behavior on permanganate oxidation. In Column 3 are recorded the observed fluorescence increments at the indicated concentration levels. For purposes of comparison, the Δ values given by a PGA standard (1 γ per ml.) at the same instrument sensitivity are recorded in Column 4.

From Table IV it can be seen that xanthopterin, isoxanthopterin, and pteroic acid interfere significantly with the determination of PGA with the direct method. When the PGA oxidation product is isolated by adsorption and elution, xanthopterin is converted to leucopterin which does not fluoresce. Isoxanthopterin is not widely distributed and is not a likely source of error. Pteroic acid, however, interferes in both methods, since it yields the same dibasic acid on oxidation as does PGA (and PGA conjugates). Similarly, high concentrations of tyrosine or tryptophan give erroneously high results in both methods. The products resulting from the oxidation of these amino acids are not completely removed in the adsorption-elution step. (They do not interfere, however, in the modified procedure described in the section covering the application of the method to natural products.) Histidine does not interfere.[2]

Pteroylglutamic acid can be determined in admixture with xanthopterin by a modification of the direct oxidation technique. The method depends on the preliminary conversion of xanthopterin to leucopterin by incubation with the enzyme, xanthine oxidase. Subsequent permanganate oxidation then gives the fluorescence increment characteristic of the PGA component of the mixture.

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[2] Vitamin B_{12} does not interfere in either procedure.

Several applications of this method are listed in Table V, where the fluorescence intensities after incubation and the increments on subsequent oxidation are listed for mixtures of PGA and xanthopterin. The data were obtained as follows: 10.0 ml. aliquots of standard solutions containing the indicated concentrations of xanthopterin and PGA were buffered to pH 6.3 by the addition of 1.0 ml. of 0.1 M phosphate buffer. The solution was then incubated for 60 minutes at 37° with 0.04 ml. of a xanthine oxidase preparation. (The enzyme was prepared according to the method of Ball (5) and was suspended in 4 M $(NH_4)_2SO_4$. 0.04 ml. of this suspen-

Table IV

Effect of Permanganate Oxidation on Fluorescence

Substance (1)	Concentration (2)	Increment on oxidation (3)	Δ, 1 γ PGA per ml. (at this sensitivity) (4)
	γ per ml.		
Riboflavin	1	0	78
	10	0	78
Thiamine	2	0	78
	10	2	78
Nicotinic acid	2	0	78
	10	0	78
Thymine	2	0	78
	10	0	78
Uracil	10	0	78
Xanthine	10	1	78
Adenine	200	1	78
Guanine	250	1	78
DL-Phenylalanine	1000	0	78
L-Tyrosine	50	8	78
	1000	36	78
DL-Tryptophan	1000	65	78
Pyridoxine	1	1	78
	10	15	78
N^1-Methylnicotinamide	1	0	78
	10	2	78
Xanthopterin	1	−76	78
Isoxanthopterin	1	−15	11
Rhizopterin*	3.4	0	35
Pteroic acid	0.8	36	80

* Supplied by the Research Laboratories of Merck and Company, Rahway, New Jersey.

sion completed the enzymatic oxidation of 10.0 ml. of a 2 γ per ml. xanthopterin standard in 45 minutes at 37°.) Following incubation, 0.1 ml. of 2.5 M acetate buffer was added and the intensity of fluorescence was determined before and after permanganate oxidation. In Column 5 are recorded the Δ values obtained by the above procedure for the same concentrations of PGA at the same instrument sensitivity.

The full fluorescence increment characteristic of the PGA oxidation product is recovered in all cases. Enzyme-free controls treated in the same way show a fluorescence decrement on permanganate oxidation.

By determining the fluorescence decrement due to enzymatic oxidation in standard xanthopterin solutions, the method can be extended to give both the xanthopterin and PGA concentrations in a mixture. By comparing the loss of fluorescence on incubation with that of xanthopterin standards, one determines the xanthopterin concentration of the solution. The subsequent increment on permanganate oxidation is proportional to the PGA concentration.

TABLE V

Determination of PGA on Admixture with Xanthopterin

Solution (1)	Fluorescence after incubation (2)	Intensity after oxidation (3)	Increment (4)	Δ values of PGA control (5)
0.33 γ PGA + 0.33 γ xanthopterin per ml.	21	48	27	27
0.5 " " + 1.0 " " " "	25	58	33	33
1.0 " " + 1.0 " " " "	14	43	28	28
1.5 " " + 1.0 " " " "	12	47	35	35
2.0 " " + 1.0 " " " "	15	58	43	43

Application of Method—Application of Method II to water extracts of some natural products gave the results indicated in Table VI. The procedure was as follows: The weighed sample was ground with water for 5 minutes in a Waring blendor. The resultant suspension was brought to the indicated concentration and heated in a 100° water bath for 15 minutes. After filtration, 10 ml. aliquots of the clear filtrate were oxidized according to the standard procedure and passed through Florisil columns. Columns were washed with 50 ml. of 0.25 M acetate buffer and eluted with four 5 ml. portions of boiling 4 per cent sodium tetraborate solution. Eluates were titrated to pH 4 to 4.5, diluted to 25 ml., and buffered at pH 4 by the addition of 0.25 ml. of 2.5 M acetate buffer. The fluorescence intensities were determined on 1 ml. of these solutions before and after the addition of 0.1 ml. of 40 per cent NaOH.

The decrement in fluorescence intensity was then calculated. The corresponding PGA concentrations were read from the standard curves of Fig. 1.

In Column 4 of Table VI are recorded the PGA concentrations in the filtrates as determined by microbiological assay with *Lactobacillus casei.* (The method used was essentially that of Roberts and Snell (6) as modified by Olson, Fager, Burris, and Elvehjem (7).) Since both methods of analysis were applied to the filtrates obtained after heating the suspensions, the analytical data are expressed as γ per ml. rather than as γ per gm. of sample. This permits a comparison of the methods without the additional assumption that the extraction procedure was completely effective in releasing bound PGA from the tissues examined.

The results obtained by the chemical method are listed in Column 3 of Table VI. They are higher than the microbiological results in all cases, although the agreement is fair for the four samples listed. The discrepancy may be due, in part, to the presence of PGA conjugates, 6-pteridyl aldehyde, pteroic acid, or other PGA hydrolysis products in the extracts examined. (Such compounds would not produce a growth response in PGA-deficient lactobacilli.) High concentrations of tyrosine and tryptophan also interfere with the method, since their oxidation products are fluorescent and follow the recommended adsorption-elution procedure.

TABLE VI

PGA Content of Natural Extracts

Sample (1)	Concentration of suspension (2)	PGA content of filtrate, γ per ml.	
		Chemical (3)	Microbiological (4)
	gm. per ml.		
Lettuce	0.5	0.29	0.23
Carrots	0.5	0.27	0.20
Potato	0.5	0.29	0.20
Beef muscle	0.4	0.13	0.10

In certain natural extracts, therefore, a more specific isolation of the PGA oxidation product is required. This can be achieved as follows: The Florisil eluate is titrated to pH 5, brought to 25 ml., and buffered with 0.25 ml. of 2.5 M acetate buffer (pH 5.0). The intensity of fluorescence of the solution is determined before and after the addition of about 0.5 gm. of Magnasol (prewashed with acetate buffer of pH 5). The decrement in fluorescence intensity represents the specific adsorption of the PGA oxidation product. If a further check is desired, the oxidation product may be eluted from the Magnasol with four 5 ml. portions of boiling 4 per cent sodium tetraborate solution, and the fluorescence decrement between pH 4 and 14 determined as before.

Application of this modified procedure to yeast extracts gives analytical results in satisfactory agreement with the PGA concentrations as determined by microbiological assay.

The recovery of added amounts of PGA from the filtrates, according to Method II, is essentially complete in all cases. In Table VII are recorded the fluorescence decrements ($-\Delta$) of the filtrates and of "recovery" samples containing the indicated added amounts of PGA. The difference between the decrement observed in the "recovery" sample and that obtained in the original filtrate is given in Column 4. In Column 5 are recorded the corresponding $-\Delta$ values of PGA standards at the same final concentrations and at the same instrument sensitivity. The percentage recovery of the added PGA is given in Column 6. The recoveries of added amounts of PGA from the filtrates are greater than 90 per cent in all cases.

TABLE VII

Recovery of Added PGA in Extracts of Natural Products

Sample (1)	PGA added per 10 ml. extract (2)	Fluorescence observed (3)	Decrement difference* (4)	$-\Delta$ of PGA control (5)	Recovery (6)
	γ				*per cent*
Lettuce	0	11.5			
	4	21.0	10.5	10.5	100
	8	30.5	19.0	20.5	93
	10	37.0	25.5	27.0	95
	20	56.0	44.5	46.5	96
Beef muscle	0	3.5			
	2.5	10.3	6.8	7.0	97
	5.0	18.0	14.5	14.5	100
	10	29.0	25.5	27.0	94
	20	50.5	47.0	46.5	102
Salmon	0	35.0			
	12	65.5	30.5	31.5	97
Carrots	0	8.5			
	3	17.0	8.5	8.5	100
Onion	0	27.0			
	4	37.0	10.0	10.5	95
Milk	0	38.0			
	3	46.0	8.0	8.5	94

* Average of three determinations.

DISCUSSION

The methods described do not include an efficient procedure for extracting bound PGA from tissues. Preliminary experiments indicate that PGA is stable to autoclaving (15 minutes at 15 pounds pressure) in neutral or faintly acid solutions (pH $\geqslant$ 4.5) and in 0.005 to 0.01 N NaOH. It is partially hydrolyzed by autoclaving in 0.1 N alkali and almost completely hydrolyzed in 0.1 N acid. The extraction procedures recommended for the microbiological method by Olson *et al.* (7) (heating in a boiling water bath for 3 minutes at pH 4.5 or 7; autoclaving at pH 4.5 for 5 minutes) do not result in hydrolytic destruction of the vitamin as followed fluorometrically. However, it cannot be concluded that such methods make possible the extraction of the total PGA content of natural materials. The effects of hydrolytic enzymes and the conjugases of hog kidney (8) and chicken pancreas (9) are currently being investigated.

Solutions of PGA are not stable to prolonged standing or to exposure to direct sunlight. Since pure PGA does not fluoresce, it is likely that the observations of Villela (2) represent partial hydrolysis of his synthetic PGA standards.

Inspection of the recovery data and internal standardization curves for lettuce and beef muscle extracts indicates that the method is accurate within about ±6 per cent. The precision in duplicate determinations by both procedures is about ±3 per cent.

SUMMARY

1. By permanganate oxidation pteroylglutamic acid (PGA) is converted to 2-amino-4-hydroxypteridine-6-carboxylic acid which fluoresces strongly at 470 mμ when irradiated with light of wave-length 365 mμ. The increment in intensity of fluorescence is directly proportional to PGA concentration over a wide range. (*a*) In the absence of fluorescent pigments which are altered by permanganate oxidation, the reaction may be standardized to permit the determination of PGA in concentrations between 0.01 and 10 γ per ml. (*b*) When interfering pigments are present, the oxidation product may be isolated chromatographically. Its concentration is then determined by the decrease in intensity of fluorescence between pH 4 and 14.

2. The variation of fluorescence intensity of the PGA oxidation product with pH and salt concentration is described.

3. Results obtained by application of the fluorometric method to several natural extracts are compared with the analytical values obtained by microbiological assay with *Lactobacillus casei*.

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LIVER GLUTAMINASES

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The enzymatic desamidation of glutamine in rat liver digests is considerably accelerated by added α-keto acids (1–5) and by added phosphate, arsenate, and sulfate (6, 7). Aqueous rat liver extracts brought to pH 5.5 or heated at 50° for a brief period of time and then neutralized or cooled lose nearly entirely the capacity to desamidate glutamine in the presence of added phosphate but not in the presence of added pyruvate (4). On the basis of these observations, it was suggested (4) that the desamidation of glutamine in rat liver was accomplished by two different enzyme systems, one activated by phosphate, the other by pyruvate. The proof of this possibility could, however, only be obtained by a physical separation of the enzymes concerned. This has been accomplished by fractionation of rat liver into two separate enzyme preparations, one of which possesses the capacity to desamidate glutamine in the presence of phosphate, but not in the presence of pyruvate (glutaminase I), and the other to desamidate glutamine in the presence of pyruvate, but not in the presence of phosphate (glutaminase II). During the course of these experiments a third fraction was obtained which was active in the desamidation of asparagine but not in that of glutamine. This partially purified enzyme was found to be active only in the presence of a variety of salts, and its description will form the basis of a future communication.

Methods

Assays—The fractionation procedure was followed by assays performed under the following conditions: (*a*) The pH of the buffer was optimal (pH 8.0 for glutaminase I, pH 7.7 for glutaminase II), (*b*) the concentration of the active protein and substrate chosen was such that the rate of hydrolysis was proportional to enzyme concentration, and (*c*) if cosubstrates or activators were used, the molarity chosen corresponded to their maximum accelerating effect on the rate of desamidation (1–7).

Analytical Methods—Desamidation was followed by the rate of ammonia formation as determined by nesslerization after aeration into sulfuric acid traps or by the micro diffusion technique. The colors were compared with a universal Coleman spectrophotometer at a wave-length of 4600 A. All determinations were made in duplicate and checked to within 2 or 3 γ of ammonia nitrogen. The relative activity of the various fractions is expressed as the micromoles of amide nitrogen hydrolyzed in 1 hour by a quantity of preparation containing 1 mg. of nitrogen.

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The L-glutamine employed was donated by the Lederle Laboratories, American Cyanamid Company. When hydrolyzed at 100° for 5 minutes in 5 N HCl, 95 per cent of theoretical amide nitrogen was recovered.

Pyruvic acid was obtained from the Eastman Kodak Company, and the sodium salt was crystallized from an alcoholic sodium hydroxide solution (7).

General Procedure—If not otherwise stated, the incubation mixtures consisted of 4 cc. containing 14.6 μM of glutamine, 46 μM of sodium pyruvate, or 100 μM of sodium phosphate, and from 0.5 to 1.0 mg. of protein nitrogen. The digests were buffered with 0.015 M veronal-acetate buffer. Blanks were run in the presence of the amide alone without added activator, but in the partially purified preparation their values were generally negligible (1 to 3 γ of ammonia).

TABLE I

Recovery of Original Activity of Crude Rat Liver Homogenate after Centrifugation

Fraction	Preparation No.	Glutaminase I	Glutaminase II
		per cent	*per cent*
Supernatant	1	39	80
	2	34	60
	3	25	
Sediment	1	66	20
	2	56	16
	3	80	

Separation of Glutaminases I and II—The essential basis of the separation of the two glutaminases lies in the fact that when a homogenate of fresh rat liver is subjected to high speed centrifugation glutaminase I activity is found largely in the sediment, while glutaminase II activity is found largely in the supernatant.

The homogenates of rat liver are prepared by mixing the fresh tissue in the Waring blendor for 3 to 5 minutes with 3 to 4 times the weight of water and crushed ice. The pH of this homogenate is then brought to a value of 7.5 to 8.0 with dilute NaOH. When this homogenate is centrifuged for 1 hour at 60,000 × g in a refrigerated Sharples centrifuge, the distribution of enzymatic activity between sediment and supernatant occurs in a manner shown in Table I. The sediment is worked up for the preparation of glutaminase I.

Preparation of Glutaminase I—The sediment from the above centrifugation is dispersed in several volumes of water, allowed to stand for 1 hour, and then centrifuged in the refrigerated Sharples centrifuge. The supernatant is discarded. This procedure is repeated in the presence of 0.05 M KCl. For assay, the final sediment is suspended in water. Approximately 25 to 30 per cent of the original activity of the crude homogenate is recovered. Some of the activity may have been lost in the different supernatants, owing possibly to incomplete sedimentation of the particles at each step, and possibly to partial extraction of the enzyme.

On a nitrogen basis, the activity is concentrated about 3-fold. No attempt was made to purify the preparation further, since its glutaminase activity was accelerated by added phosphate but not by added pyruvate, and hence it possessed glutaminase I activity and no glutaminase II activity.

Preparation of Glutaminase II—The supernatant from the original centrifugation of the crude liver homogenate can be further fractionated to yield glutaminase II. It has been found, however, that a more efficient and cleaner fractionation is achieved through a preliminary treatment of the homogenate with ethanol at low temperature. The crude homogenate is therefore treated at −10° with cold 50 per cent ethanol to a final alcohol concentration of 20 per cent. The mixture is permitted to stand at −10° for 12 hours and then centrifuged in the refrigerated Sharples centrifuge. The sediment is discarded.

The supernatant is then brought to pH 5.3, allowed to stand for 2 hours, and then centrifuged in the Sharples centrifuge. The supernatant is discarded. The sediment, which contains most of the glutaminase II activity, is mixed with 3 volumes of water and crushed ice in a Waring blendor, and the mixture allowed to stand for 2 hours at 0° and then centrifuged in an International refrigerated centrifuge for 30 minutes. The enzyme activity is now found in the supernatant.[1]

This supernatant is adjusted to pH 6.5 and solid KCl is added to a final concentration of 0.05 M. An equal volume of chilled 70 per cent ethanol is added, and the alcoholic mixture allowed to stand for 12 hours at −15°. The mixture is centrifuged in the Sharples centrifuge and the supernatant discarded.

The sediment from the above operation is suspended in water at pH 6.8, and allowed to stand for several hours to extract the enzymatic activity.

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[1] The sediment from this operation contains asparaginase activity. It is washed in 0.04 N acetate buffer at pH 5.3, and then extracted overnight with 3 times its volume of 0.05 M KCl at pH 6.2. After removal of the insoluble material by centrifugation the supernatant may be employed for the preparation of asparaginase free from glutaminase.

After centrifugation in the Sharples centrifuge, the sediment is discarded and the chilled supernatant treated with an equal volume of chilled 70 per cent ethanol. After standing for 12 hours at −15°, a small, inactive precipitate is discarded by centrifugation. The supernatant is treated with solid KCl to a concentration of 0.05 M, brought to pH 5.3, and allowed to stand for 2 hours. The mixture is centrifuged in the refrigerated Sharples centrifuge and the supernatant discarded.

The sediment is suspended in 4 volumes of cold water and allowed to extract in the cold. After centrifugation, the sediment is discarded, and the supernatant adjusted to pH 6.6 and treated with 75 per cent ethanol to a final alcohol concentration of 12.5 per cent. A small inactive precipitate is discarded. The pH is then adjusted to 5.4 and an abundant active precipitate appears, which is immediately centrifuged in the Sharples centrifuge. This sedimented precipitate is extracted with 4 volumes of water, centrifuged, and the supernatant designated as Fraction IIa (relative activity, 8.3; 20 per cent of initial activity of crude homogenate recovered). If the alcohol concentration of this supernatant is increased to 30 per cent, a precipitate forms which, after centrifuging and solution in water, yields a fraction designated Fraction IIb (relative activity, 10.4).

TABLE II

Activity of Purified Enzyme Preparations

Preparation	Salt added*	Glutamine hydrolyzed per hr. per mg. N
		μM
Original homogenate	None	0.15–0.25
	Phosphate	0.65–0.95
	Pyruvate	0.45–0.75
Glutaminase I	None	0.45
	Phosphate	1.93
	Pyruvate	0.0
" II (Fraction IIb)	None	0.0
	Phosphate	0.0
	Pyruvate	10.4

* Phosphate added, 100 μM; pyruvate added, 46 μM.

Two other preparations obtained by similar procedures possessed relative activities of 5.4 and 6.5 (Fractions I and III respectively).

The absolute separation of glutaminase I and glutaminase II activity is shown in Table II.

Studies on Glutaminase II

Effect of pH—The effect of pH on desamidation of glutamine by glutaminase II was investigated. The maximum rate of desamidation was found to be near pH 7.7. Below pH 5 and above pH 10 only about 10 per cent of the maximum activity was noted. The pH optimum noted for digests with crude tissue extracts was 7.2 (5) (Fig. 1).

Time Curve of Reaction—Experiments in which the desamidation of glutamine was followed for a period of several hours revealed that (*a*) the substrate is almost completely hydrolyzed in 3 hours, and (*b*) the rate of hydrolysis is practically constant until 50 per cent of the substrate is split (Fig. 2).

Effect of Enzyme Concentration—The rate of hydrolysis was proportional to protein concentration up to a value of approximately 600 γ of protein nitrogen (Fig. 3). The flattening of the curve at higher protein concentrations might be due to the need for more pyruvate for maintenance of optimal activity, as the amount of enzyme becomes excessive.

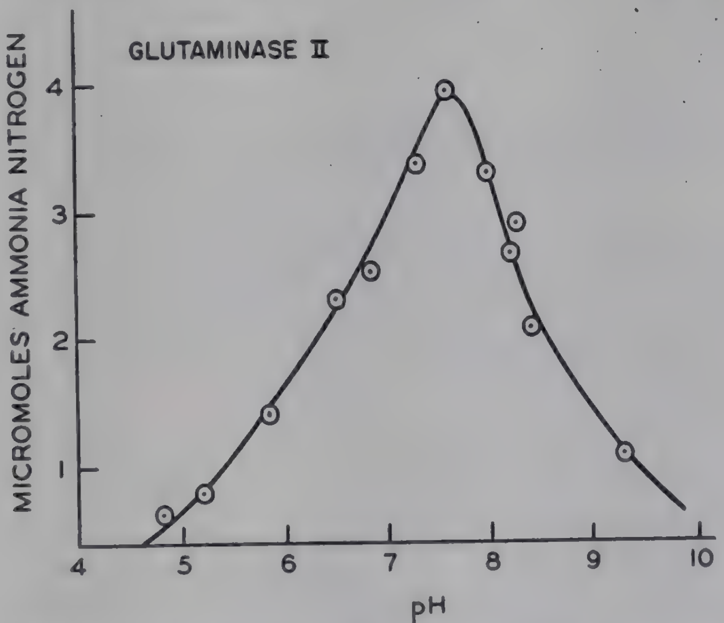

FIG. 1. Effect of pH on glutaminase II activity. The digests consisted of a total volume of 5 cc. containing 14.5 μM of glutamine, 23 μM of pyruvate, 1.25 mg. of protein nitrogen in 0.024 M veronal buffer (Fraction I).

Order of Reaction—Enzymatic activities were calculated from the initial reaction rates, obtained from a family of curves of hydrolysis against time determined at a low glutamine concentration and at different pyruvate concentrations. These data fitted a first order curve and rate constants were calculated for each pyruvate concentration from the equation

$$K = \frac{1}{t} \log \frac{100}{100 - a}$$

where K = the rate constant, a the per cent of total available substrate hydrolyzed in time t (Table III).

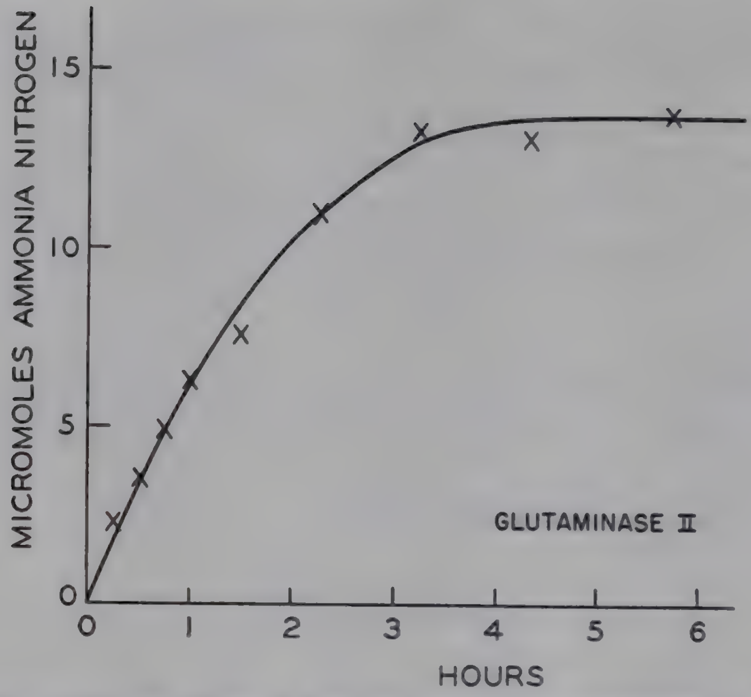

Fig. 2. Relation between time of incubation and desamidation of glutamine by glutaminase II. The digests consisted of a total volume of 6 cc. containing 14.6 μM of glutamine, 23 μM of sodium pyruvate, 0.871 mg. of protein nitrogen in 0.010 M veronal buffer at pH 7.7 (Fraction I).

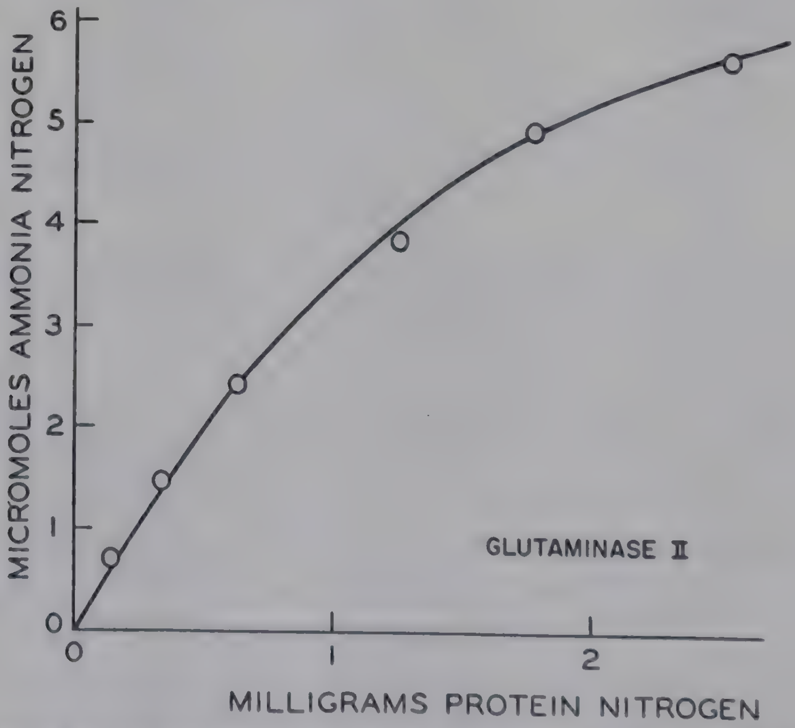

Fig. 3. Effect of enzyme concentration on glutamine desamidation by glutaminase II. The digests consisted of a total volume of 6 cc. containing 14.6 μM of glutamine, 23 μM of sodium pyruvate, and protein Fraction III in 0.01 M veronal-acetate buffer at pH 7.7. Incubation period 60 minutes.

Influence of Glutamine Concentration—Influence of glutamine concentration is illustrated in Fig. 4. The data show that saturation of enzyme

TABLE III

*First Order Reaction Constants for Glutaminase II Activity at Low Glutamine Concentration**

Pyruvate, 0.48 μM		Pyruvate, 1.37 μM		Pyruvate, 2.92 μM	
Time	*K*	Time	*K*	Time	*K*
min.		*min.*		*min.*	
17	0.00143	14	0.00307	15	0.00470
33	0.00112	33	0.00300	30	0.00457
63	0.00129	64	0.00340	61	0.00458
129	0.00100	131	0.00386	125	0.00443
182	0.00112	185	0.00304	189	0.00245
Mean	0.00119		0.00327		0.00456

* The digests consisted of 2 cc. containing 1.4 μM of glutamine and the indicated amounts of pyruvate in 0.015 M veronal-acetate buffer at pH 7.7 plus water to bring to volume. Fraction IIa, 0.418 mg. of protein N.

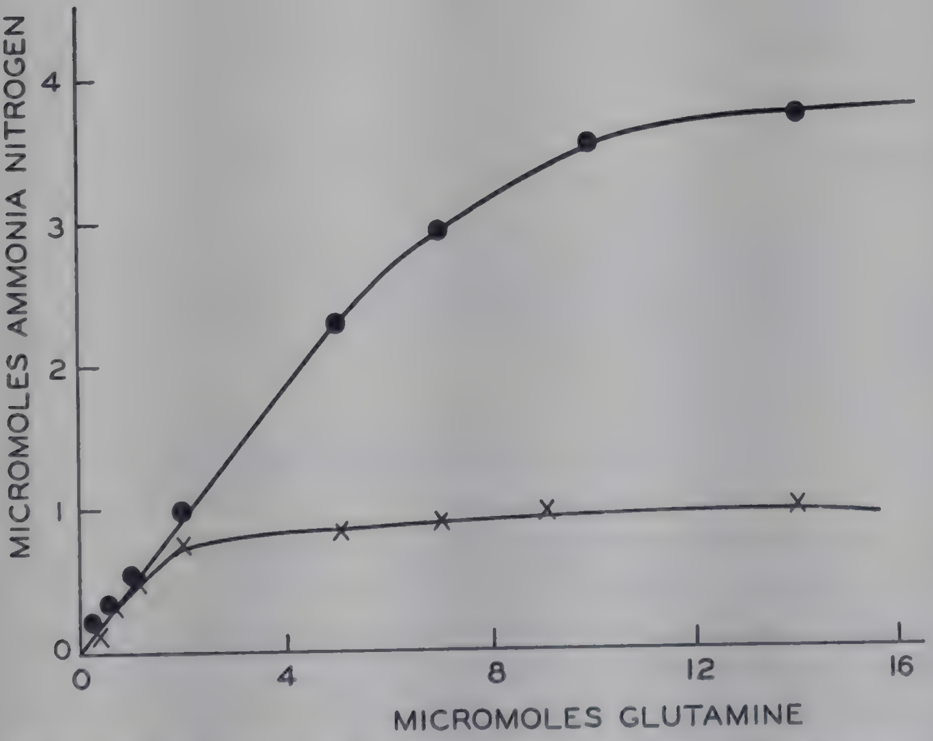

FIG. 4. Effect of substrate concentration on glutaminase II activity. The digests consisted of 2 cc. containing 46 μM (●) or 4.6 μM (×) of sodium pyruvate plus protein Fraction III in 0.150 M veronal-acetate buffer at pH 7.7; 1 mg. of protein nitrogen. Incubation period 90 minutes.

towards substrate occurs at a glutamine concentration approximately 5 times lower for the digest containing 10 times less pyruvate.

Pyruvate Recovery—It has been reported (1–5) that the pyruvate added to digests of tissue and glutamine could be nearly quantitatively recovered at the end of the reaction. Repetition of these experiments with purified glutaminase II and low pyruvate concentrations revealed no appreciable disappearance of the keto acid.

These experiments were performed with 2 cc. digests containing 7.3 μM of glutamine, 4.53 μM of sodium pyruvate, and 1.05 mg. of protein nitrogen (Fraction IIb) in 0.015 M veronal-acetate buffer at pH 7.7. Sodium pyruvate was determined as the 2,4-dinitrophenylhydrazone as previously described (6). At 0, 90, and 190 minutes of incubation, 4.48, 4.12, and 4.08 μM of pyruvate were recovered, respectively.

TABLE IV

*Effect of Inhibitors on Glutaminase II**

Substance added	Glutamine hydrolyzed	Inhibition
	μM	per cent
None	2.6	
Potassium cyanide	0.0	100
Sodium iodoacetate	0.0	100
" thioglycolate	1.95	25
" azide	1.85	29
Cysteine	0.56	77
Sodium fluoride	2.55	0
Potassium iodide	1.83	30

* The digests consisted of 2 cc. containing 7.3 μM of glutamine, 23 μM of pyruvate, 50 μM of inhibitor, and 0.192 mg. of protein N (Fraction IIa) in 0.015 M veronal-acetate buffer at pH 7.7, plus water to bring to volume. Incubation period, 90 minutes at 37°.

Approximately 90 per cent of the added pyruvate was recovered at the end of the experiment. This is about the same recovery obtained after incubation of pyruvate with a preparation containing no glutamine.

Inhibition Studies—In order to characterize this enzymatic system further certain reagents were tested for their possible inhibitory effect. The results of these studies are shown in Table IV. Inhibition by KCN and iodoacetate is at least partly reversible, since dialysis of the solution of enzyme plus inhibitor for 3½ hours restored 40 per cent of the activity in the case of KCN and 75 per cent with iodoacetate. Complete inhibition was noted with 0.012 M KCN.

Action on Isoglutamine and Asparagine—Glutaminase II has very little effect on asparagine. Isoglutamine, which is very rapidly hydrolyzed by crude liver extracts (2), is also hydrolyzed by glutaminase II (Fraction IIb). The relative activity found for this substrate was 4.1 under conditions identical with those given in Table II. No appreciable acceleration of the desamidation of this α-amide was found in the presence of pyruvate.

Dehydropeptidase Activity—As a possible condensation between glutamine and the keto acids to form an intermediary dehydropeptide had previously been suggested as an explanation for this enzymatic reaction (1), it was thought of interest to test glutaminase II preparations for dehydropeptidase activity. 2 cc. digests containing the following were incubated for 60 minutes: 6.25 μM of dehydropeptide and 0.195 mg. of protein nitrogen of Fraction IIb in 0.015 M veronal-acetate buffer at pH 7.7. It was found that, whereas glycyldehydroalanine and chloroacetyldehydroalanine are relatively slightly hydrolyzed (relative activity, 5.0 in both cases), alanyldehydroalanine is still split at an appreciable rate (relative activity, 17). However, no definite conclusions can be drawn from these facts, because the possible dehydropeptide arising from the condensation of glutamine and pyruvic acid would not necessarily be hydrolyzed by the same enzyme as any of the dehydropeptides tested.

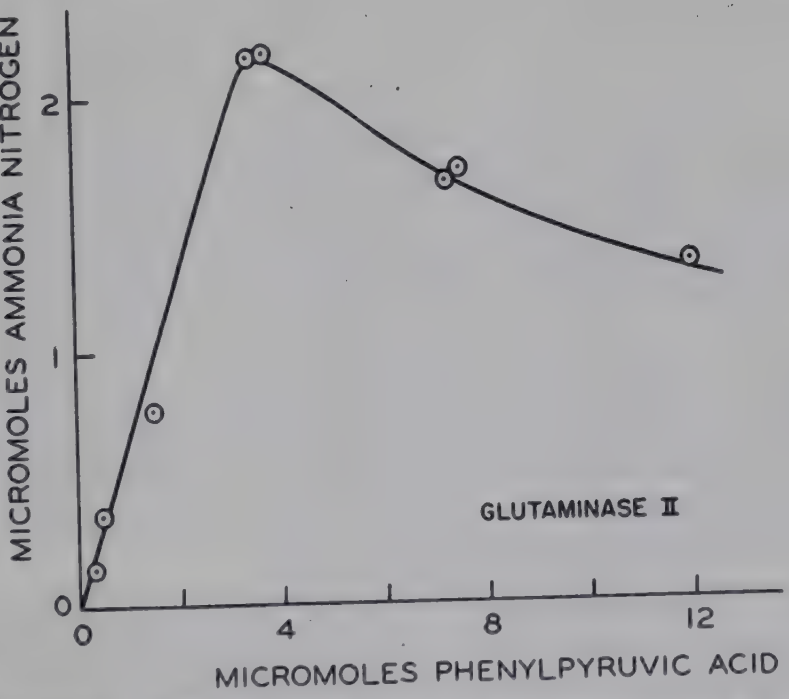

Fig. 5. Influence of phenylpyruvic acid on the desamidation of glutamine by glutaminase II. The digests consisted of 2 cc. containing 7.3 μM of glutamine and 0.167 mg. of protein nitrogen (Fraction IIb).

Phenylpyruvate—It had previously been found that sodium phenyl pyruvate was capable of accelerating the desamidation by crude liver extracts (5). The same phenomenon was observed with the partially purified preparation (Fig. 5).

DISCUSSION

Fractionation experiments herein reported have led to the separation of two different enzyme systems from rat liver which are capable of hydrolyzing the amide group of glutamine under different conditions. One system is active in the presence of phosphate, the other in the presence of pyruvate. The systems have been designated glutaminase I and glutaminase II, respectively.

Glutaminase I—In earlier studies it had been shown that the rate of desamidation of glutamine was considerably accelerated in the presence of phosphate, arsenate, sulfate, and ethyl phosphonate in liver, spleen, and brain extracts (6, 7). Glutaminase I, which is apparently bound to insoluble tissue particles in the liver and kidney, hydrolyzes glutamine more actively in the presence of added phosphate than in its absence. It is possible that similar preparations could be obtained from brain and spleen extracts and that sulfate, arsenate, and ethyl phosphonate might also be found to be activating agents. This, however, awaits further study. It is difficult to decide whether the activity of crude tissue extracts towards glutamine in the absence of added salt is due to activation by tissue electrolytes. Extensive dialysis (7) or washing of the active insoluble material does not result in complete loss of activity in the absence of added phosphate. It is possible that inorganic phosphate liberated from tissue components, such as nucleic acid, during the course of incubation may serve as an activator in initially phosphate-free media.

Glutaminase II—Glutaminase II activity appears to be an exclusive liver function. It has been found only in hepatic tissues, whether fetal, adult, or neoplastic (4). The enzyme is water-soluble and when partially purified is not active in the absence of added α-keto acids. Pyruvate, phenyl pyruvate (5), and α-ketoisocaproic acid (8) have been found to activate the enzyme in crude extracts. No activation was found with crude extracts with added pyruvoylglycine, lactic acid, or levulinic acid. The desamidation of isoglutamine, like other α-amides, is not accelerated by added pyruvate (3). The reaction thus seems to concern the γ-amide group of the amino acid amide and the α-keto group of the keto acid, which in this case appears to play the rôle of a cosubstrate and is fully recovered at the end of the reaction.

The mechanism of activation of glutaminase by pyruvate is not yet clear. The hypothesis of Greenstein and Carter (1), involving a preliminary condensation of glutamine and keto acid on the surface of the enzyme to form a susceptible dehydropeptide, followed by enzymatic hydrolysis of this intermediate, while plausible is not yet proved. It is also conceivable that pyruvate might function as a coenzyme, and that enzymatic activity is dependent upon the formation of a reversible enzyme-pyruvate complex. Further study of the pyruvate activation of glutaminase is necessary in order to elucidate this mechanism.

SUMMARY

1. Aqueous liver extracts have been separated into two distinct fractions, designated glutaminases I and II, which are capable of hydrolyzing glutamine under different conditions.

2. Glutaminase I is activated by phosphate and not by pyruvate, and is bound to insoluble liver particles.

3. Glutaminase II is water-soluble, active in the presence of sodium pyruvate, and not in the presence of phosphate. Sodium phenyl pyruvate also activates this enzyme.

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PHOSPHATE-ACTIVATED GLUTAMINASE IN KIDNEY AND OTHER TISSUES

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Glutaminase I is the designation given the enzyme in rat liver, brain, and spleen extracts which is accelerated by added phosphate or arsenate (1–3) to distinguish it from liver glutaminase II which is active only in the presence of added α-keto acids (3–5). At a final concentration of added phosphate or arsenate of 0.01 M, the desamidation of glutamine in aqueous extracts of rat liver, brain, and spleen is considerably accelerated, whereas that in extracts of kidney is apparently unaffected (5). We have, however, subsequently noted that an appreciable phosphate and arsenate activation of rat kidney glutaminase can be demonstrated in extracts more diluted, and at concentrations of phosphate or arsenate more elevated, than those employed heretofore. The characteristics of this activation, and extension of the phenomena to other tissues in various species, are described.

EXPERIMENTAL

The L-glutamine employed was a gift of the American Cyanamid Company. Unless otherwise stated, the digests consisted of 1 cc. of fresh aqueous tissue extract, 2 cc. of 0.14 M veronal-acetate buffer, and 1 cc. of either glutamine solution at the concentration designated or distilled water. The extract was prepared by grinding the fresh tissue with sand to a paste, taking up the material in the desired quantity of distilled water, and lightly centrifuging to remove sand and tissue débris. The extract was always immediately employed. The veronal buffer contained 0.13 M sodium chloride. Sodium hydrogen phosphate or arsenate was dissolved in this buffer when desired, and the pH subsequently adjusted with either NaOH or HCl. Glutamine solutions in distilled water were prepared daily. Enzymatic activity at 37° was followed by measuring the ammonia evolved during the course of the reaction (1–5). No measurable ammonia was evolved in digests of extracts which were heated at 100° for 10 minutes, cooled, and incubated 1 hour at 37° with glutamine and phosphate (*cf.* (1)).

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Effect of Various Phosphate and Arsenate Concentrations on Rat Kidney Glutaminase I—A series of digests was prepared containing a 2 per cent rat kidney aqueous extract, with two different concentrations of glutamine, and with added phosphate or arsenate to yield various concentrations at a carefully adjusted constant pH of each digest of 8.0. The data are given in Fig. 1.

A maximum activation by added phosphate is apparently attained at 0.05 to 0.06 M concentration when 14 μM of glutamine were employed, and at 0.10 to 0.12 M concentration when 28 μM of glutamine were employed (Fig. 1). For both concentrations of glutamine, the molar ratio of added phosphate to glutamine at the point of maximum activation is about 17. For rat liver and brain, this molar ratio is about 8 (2). Unlike extracts of rat liver (1) and particularly brain (2), the glutaminase activity of which is very responsive to quite small amounts of added phosphate, extracts of rat kidney require much more added phosphate before an activation of the glutaminase activity becomes appreciable (Fig. 1). Like liver (1) and brain (2), when the activation by added phosphate of kidney glutaminase has reached a maximum, further addition of phosphate produces no further acceleration in the enzymatic desamidation of glutamine. This flattening off of the phosphate concentration-glutaminase activity curves stands in marked contrast with the pyruvate concentration-glutaminase

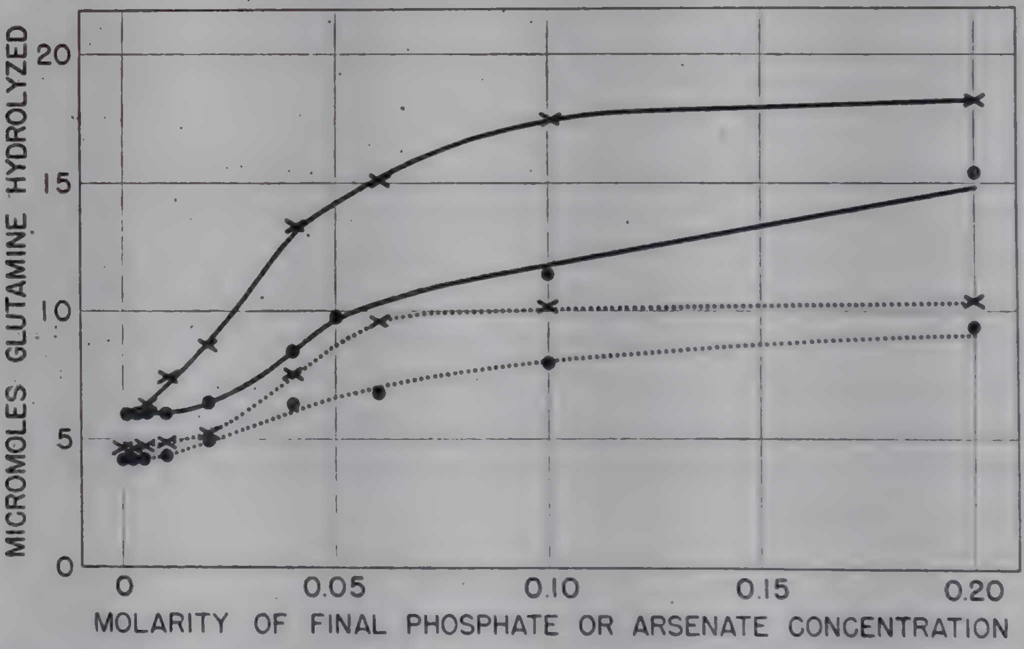

Fig. 1. Effect of phosphate and arsenate concentration on glutamine desamidation. The digests included 1 cc. of aqueous rat kidney extract containing 20 mg. of tissue. Incubation period 1 hour at 37°. pH of each digest set at 8.0. The continuous curves refer to 28 μM of glutamine, the dotted curves to 14 μM of glutamine in the digests. × = phosphate; ● = arsenate.

activity curves with rat liver extracts (5), and purified preparations therefrom (glutaminase II) (3), which decrease with increasing pyruvate after the optimal concentration has been reached.

In the absence of added phosphate, the desamidation rates for the two concentrations of glutamine used are not very different (Fig. 1). With increasing concentration of phosphate, the desamidation at each concentration of glutamine increasingly diverges until at the maximum phosphate activation the ratio of desamidation of the higher to the lower concentration of substrate is about 1.8:1, or close to the ratio of 2:1 for the original substrate concentrations (Fig. 1).

Arsenate also activates the desamidation of glutamine in rat kidney extracts (Fig. 1), but its effect is apparently weaker than that of phosphate within the concentration ranges studied. At higher concentrations, the activation by arsenate apparently approaches that of phosphate.

The acceleration of glutamine desamidation by added phosphate and arsenate occurs in digests already containing 0.065 M NaCl. Sodium nitrate or pyruvate added to a final concentration of 0.2 M produces no activation of glutaminase activity in rat kidney digests (*cf.* (5)).

Time Course of Reaction—With increasing time of incubation, and in the presence of added phosphate, the desamidation of glutamine approaches the theoretical, limiting value of 12.5 to 13 μM of substrate hydrolyzed, based upon the purity of the glutamine preparation (about 90 to 95 per cent as judged by a 1 minute HCl hydrolysis). In the absence of added phosphate the desamidation proceeds relatively slowly (Fig. 2).

pH-Activity Relation—The pH at which the optimal rate of desamidation of glutamine occurs in rat kidney digests, whether in the presence or absence of added phosphate, is close to 8.0 (Fig. 3). As in the case of liver (1) and of brain (2), the activation by phosphate does not alter the position of the pH-activity optimum.

Inorganic Phosphate Concentration in Rat Kidney Extracts—Digests consisting of 1 cc. of fresh rat kidney aqueous extract equivalent to 330 mg. of tissue, 2 cc. of veronal buffer, and 1 cc. of glutamine were incubated for 1 hour at 37°. The inorganic phosphate concentration at the beginning of the incubation period for six such digests varied from 0.0015 to 0.0019 M. After incubation, the corresponding range was 0.0021 to 0.0024 M. The kidney extracts employed in the enzyme studies reported were diluted a little over 16-fold from the above, and the phosphate concentrations were diluted approximately in proportion. The final phosphate concentration in the digests described in Figs. 1 to 3 was therefore only negligibly affected by the inorganic phosphate inherently present in the dilute extracts used.

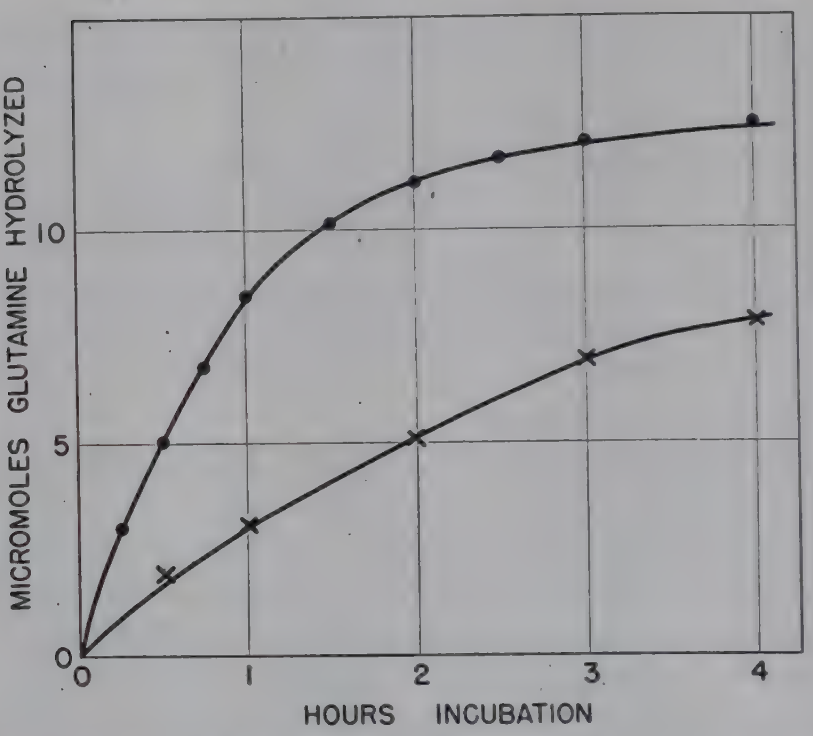

FIG. 2. Time course of phosphate activation of rat kidney glutaminase. The digests included 1 cc. of aqueous rat kidney extract containing 20 mg. of tissue. ● = added phosphate; × = no added phosphate.

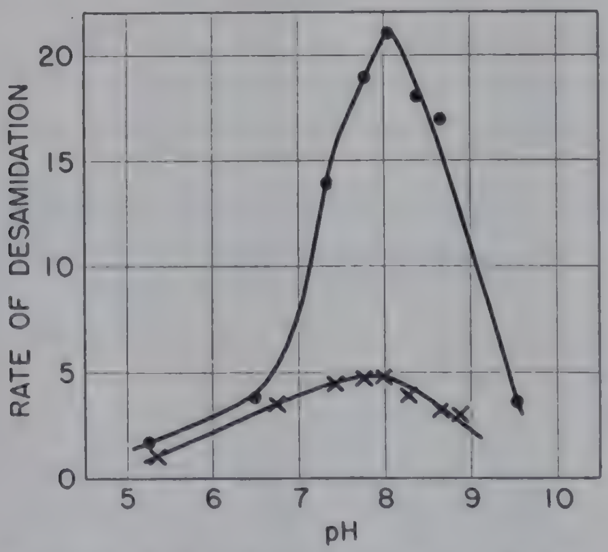

FIG. 3. pH-activity curve of phosphate activation. The digests consisted of 0.1 cc. of rat kidney extract equivalent to 33 mg. of tissue, 0.5 cc. of veronal-acetate buffer, 0.5 cc. of either water or 0.1 M phosphate, 0.5 cc. of either water or 0.014 glutamine solution, and 0.2 cc. of water. The ordinate is given in terms of micromoles × 100 of glutamine hydrolyzed per minute under these conditions. ● = added phosphate; × = no added phosphate.

Separation of Phosphate-Activatable Glutaminase (Glutaminase I) from Rat Kidney Extracts—The chilled tissue extract, equivalent to 330 mg. of fresh tissue per cc., was brought to pH 7.7 and centrifuged for 50 minutes at 50,000 R.P.M. in the refrigerated Sharples centrifuge. Practically all of the phosphate-activatable glutaminase of the extract was recovered in the sedimentable fraction. The data are given in Table I. Based upon the activity per mg. of total N in the enzyme preparations, a 2- to 3-fold increase in glutaminase I was achieved by the centrifugation. In all respects studied, these results are similar to those obtained for glutaminase I in rat liver (3).

Phosphate Activation of Glutaminase Activity in Tissues of Other Species—In order to obtain some information on how general the phenomenon of phosphate activation of glutaminase (glutaminase I activity) might be, extracts of various tissues of rats, mice, rabbits, and guinea pigs were studied in the presence and absence of 0.10 M added phosphate concentration. Suitable blanks to determine the preformed ammonia in the more concentrated tissue extracts were prepared, and appropriate corrections made in the data. The results of these studies are given in Table II, and represent data on twenty rats, forty mice, three rabbits, and four guinea pigs. The tissues from each of the rabbits and guinea pigs were studied separately. Certain tissues of the rat (spleen, brain) and all of the mouse tissues from a number of animals were pooled. The variation between tissue samples studied under the same conditions in each species was about ±10 per cent.

TABLE I

*Separation of Glutaminase I in Rat Kidney Extract**

Fraction	NH_3-N evolved	
	No PO_4	PO_4
	γ	γ
Extract	28	150
Supernatant	3	6
Pellet†	21	152

* The digests consisted of 0.2 cc. of enzyme preparation, 1 cc. of 0.014 M glutamine solution, and 1 cc. of water or 0.1 M phosphate.

† Made up to original volume.

In digests containing brain or spleen extracts from all four species, there is considerable activation of glutaminase by added phosphate. With extracts of kidney and liver, however, a marked activation occurs only in the case of the rat and the mouse. The glutaminase activity of extracts of the liver and kidney of the rabbit and guinea pig is relatively little affected by added phosphate. Phosphate added at 0.05 and 0.20 final molar concentration to extracts of rabbit and guinea pig kidney and liver gave results no different than at 0.10 M.

Fresh rabbit or guinea pig liver was homogenized with a little more than its weight of water, centrifuged lightly for 30 seconds, cold water was added to the supernatant to 3 times the weight of the tissue, and the mixture centrifuged for 1 hour in the refrigerated International centrifuge at 18,000 R.P.M. The pellet was mixed with water and brought up to the original volume. Digests consisting of 1 cc. of this mixture, 2 cc. of veronal-acetate-NaCl buffer at pH 8.3, and 1 cc. of either water or 0.014 M glutamine solution were incubated for 1 hour. In the absence of added phosphate, 1 to 2 μM of glutamine was hydrolyzed by rabbit or guinea pig liver preparations. In the presence of phosphate added to 0.1 M final concentration, 2 to 3 μM of glutamine were hydrolyzed. In the absence of phosphate, similar rat liver preparations hydrolyzed about 2 μM of glutamine, and in the presence of added phosphate about 11 μM of glutamine. Most of the phosphate-activatable glutaminase of extracts of rabbit, guinea pig, or rat liver is recoverable in the first sedimentable fraction and, on the basis of estimation of total N per unit volume, is concentrated by such sedimentation about 2- to 3-fold.

TABLE II

*Glutaminase Activity in Tissues of Various Species**

	Glutamine hydrolyzed in 1 hr., μM							
	Kidney†		Liver‡		Brain‡		Spleen‡	
	No PO_4	PO_4	No PO_4	PO_4	No PO_4	PO_4	No PO_4	PO_4
Rat	3	12	2	12	3	12	3	12
Mouse	2	12	3	13	3	12	3	13
Rabbit	1	1	2	3	1	13	4	14
Guinea pig	2	3	3	4	1	11	2	12

* The digests consisted of 1 cc. of fresh aqueous tissue extract, 2 cc. of veronal-acetate-NaCl buffer with and without sodium phosphate, and 1 cc. of either water or 0.014 M glutamine solution. Final concentration of added phosphate, 0.10 M. pH 8.0 in each digest. Temperature 37°.

† Each cc. of extract equivalent to 20 mg. of tissue.

‡ Each cc. of extract equivalent to 330 mg. of tissue.

In order to see whether there was any pyruvate-activated glutaminase activity (glutaminase II) in the livers of rabbits and guinea pigs, digests consisting of 1 cc. of aqueous liver extract equivalent to 330 mg. of fresh tissue, 1 cc. of veronal buffer at pH 6.8, 1 cc. of 0.014 M glutamine solution, and 1 cc. of either water or 0.023 M sodium pyruvate solution were incubated for 4 hours at 37°. In the absence of added pyruvate, less than 0.5 μM of glutamine was hydrolyzed. In the presence of pyruvate, 1 to 2 μM of glutamine was hydrolyzed by rabbit or guinea pig liver extracts. Under similar experimental conditions, and, in the presence of added pyruvate, extracts of rat or mouse liver hydrolyze about 10 μM of glutamine (4–6). The glutaminase of rabbit and guinea pig liver is apparently not readily activated by added phosphate or pyruvate.

The sedimentable fraction of rat liver (3) and of rat kidney (Table I) possesses significant glutaminase activity in the absence of added phosphate; addition of phosphate results in a considerable increase in this activity. The sedimentable fraction of rabbit and guinea pig liver extracts also possesses significant glutaminase activity, but addition of phosphate does not result in a very appreciable acceleration of this activity. These results suggest that either (*a*) we have not yet found the appropriate conditions to demonstrate phosphate activation of the glutaminase of rabbit and guinea pig liver preparations, or (*b*) there is a third type of glutaminase for whose activity phosphate is apparently not essential. If the second of these possibilities is true, it would suggest that the phosphate-activated glutaminase in the sedimentable fraction (glutaminase I) (3), like the pyruvate-activated glutaminase in the supernatant (glutaminase II) (3), demonstrates activity only in the presence of these respective anions. On this assumption, glutaminase I is present in liver, kidney, brain, and spleen of rats and mice and in the brain and spleen of rabbits and guinea pigs, while glutaminase II is present in the livers of rats and mice; glutaminase I is relatively weak in rabbit and guinea pig kidney and liver, and glutaminase II similarly weak in rabbit and guinea pig liver. The presumably non-activatable glutaminase would be present in all tissues studied.

SUMMARY

1. Studies of the phosphate activation of glutaminase activity (glutaminase I), hitherto studied in the tissues of the rat, have been extended to include other species.

2. The glutaminase activity at pH 8.0 in aqueous extracts of rat and mouse kidney, liver, brain, and spleen and of rabbit and guinea pig brain and spleen is greatly increased by added phosphate. Under the same conditions, the glutaminase activity of extracts of rabbit and guinea pig kidney and liver and of the sedimentable fraction of rabbit and guinea pig liver is only slightly increased by added phosphate.

3. Studies of the effect of various phosphate concentrations on the desamidation of glutamine in extracts of rat kidney revealed that a higher concentration of phosphate was necessary to achieve an appreciable acceleration of desamidation than was the case in extracts of brain or liver. The concentration of phosphate required to yield maximum acceleration

of glutaminase activity appeared to be proportional to the concentration of glutamine used.

4. Arsenate also accelerated glutaminase activity in rat kidney extracts but appeared to be relatively less effective than phosphate.

5. The pH for maximum glutaminase activity in rat kidney extracts, in the presence or absence of added phosphate, was close to 8.0.

6. Most of the phosphate-activatable glutaminase kidney extract can be recovered in the pellet after submitting the extract to a single high speed centrifugation. A 2- to 3-fold increase in activity was thereby achieved.

7. The distribution of glutaminase activity among the tissues of various species is discussed.

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PREPARATION OF L- AND D-ALANINE BY ENZYMATIC RESOLUTION OF ACETYL-DL-ALANINE

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Studies under way in this laboratory on the separation and characterization of intracellular peptidase systems of tissues have required the availability of large stocks of optically pure amino acids for the synthesis of appropriate substrates. The observation that aqueous extracts of rat kidney rapidly hydrolyzed one of the isomers of acetyl-DL-alanine (1) suggested a simple, convenient, and rapid method of preparing L- and D-alanine in quantity and with a high degree of optical purity.

Other enzymatic methods of resolution of racemic amino acids include that of asymmetric synthesis with aniline to resolve DL-phenylalanine (2), DL-glutamic acid (3), and DL-methionine (4), and by the use of D-amino acid oxidase to obtain L-alanine from DL-alanine (5, 6). The last method obviously involves the loss of D-alanine. Studies by du Vigneaud and coworkers (7) have shown that acetyl-D-phenylaminobutyric acid is not hydrolyzed in the body of the rat.

EXPERIMENTAL

Preparation of L- *and* D-*Alanine*

Acetyl-DL-alanine was prepared by heating 1 mole of DL-alanine with 1.5 moles of acetic anhydride and 12 moles of glacial acetic acid for 2 hours on the steam bath (*cf.* (8)). The resulting solution was evaporated *in vacuo* to a syrup, and the residue treated several times with water. After the final evaporation, the residue was dissolved in the minimum amount of acetone. On chilling and scratching, acetyl-DL-alanine rapidly crystallized. The product was crystallized again from acetone and separated in the form of prisms; m.p. 136°; N found, 10.7 per cent; calculated, 10.7 per cent. The yield of pure product was about 40 per cent.

A fresh hog kidney preparation was employed as the enzyme source. The initial crude homogenate of this tissue hydrolyzed 350 μM of acetyl-DL-alanine per hour per mg. of N at a substrate concentration of 0.5 M, as compared with a similar homogenate of rat kidney which, under the same conditions, hydrolyzed 24 μM of acetyl-DL-alanine. A moderate concentration of enzymatic activity in the hog kidney homogenate wa achieved in the following manner: 4 kilos of fresh frozen hog kidneys wer decorticated, and 3280 gm. of cortex ground in a Waring blendor and suspended in 8200 cc. of cold, distilled water. After being stirred for 1 hou in the cold and filtered through gauze, the extract was chilled, and 7 per cent alcohol was added to a concentration of 15 per cent. After standing for 12 hours at $-6°$, the mixture was centrifuged at 2600 R.P.M. in refrigerated centrifuge for 45 minutes. The supernatant was taken to 3 per cent alcohol, adjusted to pH 6.3, and allowed to stand for 12 hours a $-15°$. The mixture was then centrifuged in a Sharples refrigerated centrifuge at 50,000 R.P.M. The pH of the supernatant was lowered to 5.1 and the mixture allowed to stand for 12 hours. On centrifuging at 260 R.P.M., a pellet of material was obtained which hydrolyzed acetyl-DL-alanine at the rate of 1400 μM per hour per mg. of N.

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The resolution was carried out in a mixture of 715 gm. of acetyl-DL-alanine, 360 cc. of 28 per cent ammonium hydroxide, and sufficient wate to bring the volume to 4 liters. The pH was 7.9. 1000 cc. of a suspensio of the enzyme containing 1.5 gm. of protein N were added, and the mixtur was incubated at 37°. After 4 hours, determinations by the Van Slyk gasometric procedure revealed that about 80 per cent of the susceptibl form of the racemic substrate was hydrolyzed. 500 cc. more of the enzym suspension were added. Assays at the 12th hour of incubation showe 100 per cent hydrolysis of the susceptible form. No further hydrolysi was noted at the 14th and 16th hours of incubation.

The digest was then treated with 360 cc. of glacial acetic acid to brin the pH to 4.5, and 15 gm. of norit were added. After shaking and subsequent filtration, a nearly colorless filtrate was obtained. This wa evaporated *in vacuo* to a volume of 1 liter at a temperature never exceedin 35°. 2.5 volumes of absolute alcohol were added and the mixture was allowed to stand for 12 hours. The precipitate, which was nearly pur L-alanine, was filtered at the pump and washed thoroughly with alcoho until free from ammonia. The combined filtrate and washings, whic contained acetyl-D-alanine, were set aside. The preparation of L-alanine which weighed 209 gm. when dried (theory, 245 gm.), was recrystallize from a water-alcohol mixture. It separated as pure white clusters of fin needles. Yield, 169 gm. 9 gm. were recovered from the mother liquo making a total yield of 73 per cent of the theory. $[\alpha]_D^{23} = +14.4°$ (1.61 gm. dissolved in 25 cc. of 1.0 N HCl); N 15.7 per cent; calculated, 15. per cent. The method also revealed that the enzymatically susceptib isomer of acetyl-DL-alanine is the acetyl-L-alanine.

The combined alcoholic filtrates from the L-alanine preparations we acidified with HCl to pH 2.5 and allowed to stand several hours in the i chest. The precipitate, which was chiefly ammonium chloride, was removed. The filtrate was evaporated *in vacuo*, when more ammonium chloride separated on addition of acetone. This treatment was repeated until no further precipitate appeared on addition of acetone. The oily residue was dissolved in 3 liters of acetone and the solution treated with 6 liters of ether. On standing in the ice chest overnight, an oily layer precipitated which was removed and discarded. The ethereal supernatant solution was evaporated to a syrup of nearly pure acetyl-D-alanine. Attempts to induce crystallization were fruitless. The residue was therefore taken up in 10 volumes of 48 per cent HBr and the solution refluxed for 6 hours. Analysis showed that 95 to 100 per cent of the acetyl-D-alanine was thereby hydrolyzed. The solution was evaporated *in vacuo* and the residue distilled many times with water to remove excess acid. Concentrated ammonium hydroxide was added with cooling to pH 5.0, and the mixture treated with absolute alcohol to 80 per cent. The dense, white precipitate of nearly pure D-alanine was filtered at the pump, washed with alcohol until free from ammonia, and recrystallized from water-alcohol in the form of white, fine needles. Yield, 94 gm. $[\alpha]_D^{23} = -14.4°$ (1.5917 gm. dissolved in 25 cc. of 1.0 N HCl); N 15.7 per cent; calculated, 15.7 per cent.[1]

The simple method described above leads to preparations of L- and D-alanine of high optical purity. The values noted for specific rotations of +14.4° and −14.4°, respectively, compare favorably with those reported in the literature. Clough (9) reported a value of +14.7° for L-alanine in 0.97 N HCl at 15°, and Pacsu and Mullen (10) a value of +14.6° in 1.0 N HCl at 20° (*cf.* (11)). Dunn *et al.* reported a value of +13.77° in 6.0 N HCl at 25° for L-alanine and −13.60° for D-alanine under the same conditions (12). $[\alpha]_D^{20} = -14.5°$ for D-alanine was reported by Pacsu and Mullen (10). All of the other values in HCl, listed by Dunn *et al.* from the literature (12), are generally lower in magnitude than those cited.

Preparation of Chloroacetyl-L-alanine and Chloroacetyl-D-alanine

Kidney extracts hydrolyze only one isomer of chloroacetyl-DL-alanine (1) and a method has been devised whereby optically pure preparations of chloroacetyl-L-alanine and chloroacetyl-D-alanine have been obtained. In the course of this study, a greater concentration of the hog kidney activit was achieved by the following procedure. 1050 gm. of hog kidney corte were ground in a Waring blendor, suspended in 4000 cc. of cold water, an stirred for 2 hours. The extract, after filtering through gauze, hydrolyze chloroacetyl-DL-alanine at a rate of 730 μM per hour per mg. of N. Alcohol was added to 15 per cent, and the mixture allowed to stand at −6 and pH 7.0 for 12 hours. After centrifuging at 2600 R.P.M., the activity the supernatant was 1285 μM. Two repetitions of this step, the first at p 6.6 and the second at pH 6.0, yielded a supernatant solution with an activity of 2340 μM. When alcohol was added to the supernatant to 30 per ce at pH 6.2, and the precipitate discarded, the activity rose to 2750 μ The pH was lowered to 5.6, and the mixture allowed to stand 12 hours −15°. The pellet of material recovered on centrifuging had an activit of 6300 μM. On suspending this in 450 cc. of cold water, adding alcoh to 15 per cent, and bringing the pH to 5.7 and the temperature to −6 a new pellet was obtained on centrifuging which had an activity of 13,40 μM of chloroacetyl-DL-alanine hydrolyzed per hour per mg. of N.

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[1] No serious attempts were made to work up the mother liquors from the various crystallizations of L- and D-alanine for further amounts of material, inasmuch as preliminary observations showed such material to have lower optical rotations. The yields, based throughout on the amount of acylated amino acid taken, are thus lower than they might be if the mother liquors had been sufficiently exploited. In such a case, the relative cost of the materials and of the investigator's time must be considered.

497 gm. of chloroacetyl-DL-alanine (13) (m.p. 125–126°; N found, 8 per cent; calculated, 8.5 per cent) were suspended in 2 liters of wate brought to pH 7.7 with NaOH, and the resulting solution made up to 390 cc. with water. 100 cc. of enzyme suspension containing 0.136 gm. protein N were added and the mixture was incubated at 37°. After 2 hou the racemic substrate was 50 per cent hydrolyzed (100 per cent of th susceptible isomer), and this figure did not change up to the 20th hour incubation.

The digest was acidified to pH 6.0, treated with norit, filtered, an evaporated *in vacuo*. It was brought to pH 2.5, evaporated *in vacuo* dryness, and extracted several times with acetone. The chloroacetyl- alanine dissolved, leaving a mixture of L-alanine and sodium chlorid This mixture was taken up in cold 2 N NaOH and chloroacylated in th usual manner (13). After crystallization from ethyl acetate-petroleu ether, the yield of chloroacetyl-L-alanine was 99 gm. (theory, 248 gm. M.p. 93°; $[\alpha]_D^{20} = -45.6°$ (0.92 gm. dissolved in 25 cc. of H_2O). N, 8 per cent; calculated, 8.5 per cent. Fischer and Schulze (14) reported melting point of 93.5–94.5° (corrected) and $[\alpha]_D^{25} = -45°$ (±0.2°) for the preparation of chloroacetyl-L-alanine.

The combined acetone extracts containing chloroacetyl-D-alanine an chloroacetic acid were evaporated *in vacuo* to dryness, and the residue w extracted with ethyl acetate and again evaporated to dryness. On chillin chloroacetyl-D-alanine separated in long prisms. The product was r crystallized from ethyl acetate-petroleum ether. The yield was 105 gr (theory, 248 gm.). M.p. 93°; $[\alpha]_D^{20} = +45.4°$ (0.956 gm. dissolved 25 cc. of H_2O). N 8.5 per cent; calculated, 8.5 per cent.

Chloroacetyl-L-alanine and chloroacetyl-D-alanine were aminated in the usual manner (13, 14) and the corresponding glycyl peptides prepared and purified by several recrystallizations. $[\alpha]_D^{22}$ for glycyl-L-alanine in water = −49.4° (0.517 gm. dissolved in 25 cc. of H_2O). N 19.2 per cent; calculated, 19.2 per cent. Fischer and Schulze (14) report a value of −50° for their preparation of glycyl-L-alanine. $[\alpha]_D^{22}$ for glycyl-D-alanine in water = +49.5° (0.498 gm. dissolved in 25 cc. of H_2O). N 19.0 per cent; calculated, 19.2 per cent.

Enzymatic Hydrolysis of Other Acylated Racemic Amino Acids by Crude Kidney Extract

The method described can presumably be applied to the preparation of the optical isomers of certain other amino acids, for only one isomer of the acylated racemic amino acids is hydrolyzed by hog kidney preparations. Acetyl-DL-valine (m.p. 148°; N found, 8.8 per cent; calculated, 8.8 per cent), acetyl-DL-leucine (m.p. 159°; N found, 8.2 per cent; calculated, 8.1 per cent), acetyl-DL-tryptophan (m.p. 205°; N found, 11.2 per cent; calculated, 11.4 per cent), chloroacetyl-DL-tryptophan (m.p. 153°; N found, 9.7 per cent; calculated, 9.9 per cent), chloroacetyl-DL-phenylalanine (m.p. 128°; N found, 5.9 per cent; calculated, 5.8 per cent), chloroacetyl-DL-serine (m.p. 123°; N found, 7.8 per cent; calculated, 7.8 per cent), acetyl-DL-glutamic acid (m.p. 172°; N found, 7.4 per cent; calculated, 7.4 per cent), and chloroacetyl-DL-threonine (m.p. 124°; N found 7.0; calculated, 7.2) were prepared by the interaction of the racemic amino acids with either acetic anhydride or chloroacetyl chloride in alkaline medium. The respective compounds were crystallized either from water or alcohol-water. Acetyl-DL-phenylalanine (m.p. 146°; N found, 6.7 per cent; calculated, 6.8 per cent), acetyl-DL-proline (m.p. 106°; N found, 8.8 per cent; calculated, 8.9 per cent), acetyl-DL-isoleucine (m.p. 160°; N found, 8.1 per cent; calculated, 8.1 per cent), acetyl-DL-methionine (m.p. 111°; N found 7.3 per cent; calculated, 7.4 per cent), acetyl-DL-histidine (m.p. 144°; N calculated 19.5 per cent for the compound with 1 mole of water of crystallization; found, 19.4 per cent), acetyl-DL-arginine (m.p. 256°; N found 25.3 per cent; calculated, 25.9 per cent) were prepared by the general procedure of Knoop and Blanco (15) (*cf.* (8)), which consists of heating the amino acid solution in a mixture of glacial acetic acid and a little more than the theoretical amount of acetic anhydride. The respective compounds were crystallized from acetone or water-acetone. For further comparison, chloroacetylglycine (m.p. 100°; N found 9.2 per cent; calculated, 9.2 per cent), chloroacetylglycylglycine (m.p. 176°; N found 13.4 per cent; calculated, 13.3 per cent), and acetylglycine (m.p. 203°, N found 11.9 per cent; calculated, 12.0 per cent) were prepared and studied. The action of hog kidney aqueous extracts on these compounds is described in Table I in terms of micromoles hydrolyzed per hour per mg. of N in the extract.

Of the acylated amino acids studied, those of methionine, glutamic acid, alanine, leucine, and serine, are rapidly hydrolyzed. Acylated valine, phenylalanine, arginine, threonine, histidine, and isoleucine are hydrolyzed at a lower rate, while acetyltryptophan is nearly completely resistant. It is therefore feasible to use crude hog kidney extract for the resolution of the isomers of racemic acylated methionine, glutamic acid, alanine, leucine, and serine, whereas for the others mentioned it would be advisable to use a highly concentrated active fraction of the extract.[2]

TABLE I

Enzymatic Hydrolysis of Acylated Amino Acids by Crude Hog Kidney Extract*

Substrate	Amount hydrolyzed per hr. per mg. N
	μM
Acetyl-DL-alanine†	161
Chloroacetyl-DL-alanine†	624
Acetyl-DL-phenylalanine	7
Chloroacetyl-DL-phenylalanine†	33
Acetyl-DL-tryptophan	0
Chloroacetyl-DL-tryptophan	5
Acetyl-DL-valine†	68
Acetyl-DL-leucine†	259
Acetyl-DL-methionine†	800
Acetyl-DL-proline	0
Acetyl-DL-isoleucine	16
Acetyl-DL-histidine	11
Acetyl-DL-arginine†	30
Acetyl-DL-glutamic acid	140
Chloroacetyl-DL-serine†	580
Chloroacetyl-DL-threonine†	80
Acetylglycine	50
Chloroacetylglycine	133
Chloroacetylglycylglycine	6

* The digests were composed of 1 cc. of a 0.05 M neutralized substrate solution, 1 cc. of 0.06 M phosphate buffer at pH 7.0, and 1 cc. of hog kidney cortex aqueous extract. Incubation temperature 38°. Activity is maintained on dialysis.

† Only one isomer hydrolyzed after prolonged incubation. The other racemic compounds were not studied because of the very low rate of hydrolysis.

The high rate of hydrolysis by crude hog kidney extracts of acetylglycine and of chloroacetylglycine is interesting. Rat kidney extracts have a relatively small effect on these compounds (1). Indeed, the acylated amino acids are probably attacked by a class of enzymes which, in view of the fact that the acylated aromatic acids are hydrolyzed much more slowly than the aliphatic acids, is evidently different from the classical, pancreatic carboxypeptidase system.

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[2] The asymmetric enzymatic hydrolysis of benzoyl-DL-alanine by taka-diastase was reported by Neuberg and Linhardt (16).

SUMMARY

1. L-Alanine with $[\alpha]_D^{23}$ in 1.0 N HCl = +14.4° and D-alanine with $[\alpha]_D^{23}$ in 1.0 N HCl = − 14.4° were obtained in yields of 73 and 39 per cent respectively by the asymmetric hydrolysis of acetyl-DL-alanine by an enzyme preparation from hog kidney.

2. A similar procedure combined with chloroacetylation, involving chloroacetyl-DL-alanine, yielded chloroacetyl-L-alanine with $[\alpha]_D^{20}$ in water = −45.6° (yield, 40 per cent), and chloroacetyl-D-alanine with $[\alpha]_D^{20}$ = +45.4° (yield, 43 per cent).

3. The possibility of asymmetric enzymatic hydrolysis of certain other acylated racemic amino acids has been demonstrated. Accordingly, the method may be extended to include the resolution of racemic amino acids other than alanine.

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AN EFFECT OF PYRIDOXINE ON BLOOD UREA IN HUMAN SUBJECTS

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In 1942 pyridoxine was related to protein metabolism (1). Since that time this vitamin or a derivative thereof has been described as a component of transaminase (2) and of decarboxylase (3). Pyridoxine has been claimed to be beneficial in the treatment of a variety of diseases. An attempt to produce a deficiency of this vitamin in a human subject failed to give evidence of any significant change (4). Pyridoxine has been used with apparent success in the alleviation of nausea and vomiting in pregnancy and also of the same condition consequent to irradiation (5, 6). Hesseltine (7) described a controlled investigation in which pyridoxine therapy was compared with placebo administration, but the comparison was based only on subjective evaluation of the condition of the subjects. To date there has been no objective evidence of an effect of pyridoxine in humans. This report deals with a significant response obtained in blood urea levels before and after administration of pyridoxine in cases of nausea and vomiting in pregnancy.

Methods

Three groups of human subjects were used: (1) non-pregnant females in apparent health who were regularly engaged in laboratory work or as hospital dieticians; the latter were on a constant protein intake for 5 days before blood samples were taken and throughout the period of study; (2) pregnant females with no apparent abnormality in the prenatal clinic of the Toronto General Hospital; (3) pregnant females exhibiting definite nausea and vomiting during the first trimester of pregnancy and who were classified clinically as showing hyperemesis gravidarum. All cases in this third group were hospitalized, and following admission they received supportive therapy consisting of intravenous administration of 5 per cent glucose solution until urinary ketosis ceased. Subsequently, these patients were given a balanced adequate diet supplying 60 gm. of protein each day. Evening sedation to insure sleep was furnished if required.

Urea estimations on samples of venous blood taken after a 12 hour fast (except as noted below) and of urine were made in duplicate by the procedure of Archibald (8); the required color estimations were carried out in a model No. 11 Coleman spectrophotometer. Urea clearances were e pressed as percentages of standard clearance, by the method of Mölle McIntosh, and Van Slyke (9).

In all cases identical dosage of pyridoxine was employed, consisting the oral administration of 40 mg. of pyridoxine hydrochloride in each 3 successive days.

In accordance with the recommendation of Hawkins, MacFarland, a McHenry (10), a study was made of the effect of a test load of an ami acid. This was done by measurement of changes in blood urea subseque to the oral administration of 30 gm. of DL-alanine dissolved in 300 cc. unsweetened canned grapefruit juice. This solvent had been shown

TABLE I

Effect of Pyridoxine on Fasting Blood Urea

Groups	No. of subjects	Average blood urea with standard deviation		Signific of diff ence du pyridox
		Before pyridoxine administration	After pyridoxine administration	
		mg. per cent	*mg. per cent*	t^*
Normal non-pregnant females	28	21.3 ± 5.4	21.1 ± 6.0	<1
" pregnant primiparae, all trimesters	40	12.4 ± 3.4	12.9 ± 3.6	<1
Hyperemesis gravidarum	17	10.3 ± 3.1	15.5 ± 2.7	2

* Calculated by

$$t = \frac{\bar{d}}{s_d\sqrt{\frac{1}{n}}}$$

where d = the average of individual differences in blood urea for n subjects, s_d = the standard deviation of the differences.

cause no detectable change in blood urea in a group of control subje For convenience, alanine was given at about 7.30 p.m., following a l evening meal containing not more than 6 gm. of protein at about 5 Blood samples were taken prior to the administration of alanine and e 2 hours during the ensuing 12 hours; this regimen provided essential period of 12 hours without food and without disturbing the custon meal pattern of the subjects.

Results

Table I contains the mean blood urea values for the three type subjects before and after administration of pyridoxine. The data that fasting blood urea was less in normal pregnant than in non-preg women, an observation which has been made frequently by others, that in cases of hyperemesis gravidarum the blood urea was decreased below the value normally characteristic of pregnancy. Blood urea was increased after the administration of pyridoxine only in subjects suffering from nausea and vomiting, and the increase changed the previously low level to one typical of normal pregnancy. It should be noted that all subjects with nausea and vomiting showed urinary ketosis throughout the test period.

TABLE II

Observations on Fasting Blood Urea

Groups	No. of subjects	Average blood urea with standard deviation	Significance of difference	
		mg. per cent	t	t^*
1. Normal non-pregnant females	50	22.1 ± 4.9	(1) *vs.* (2)	9.9
2. " pregnant, 1st trimester	50	14.7 ± 1.8	(2) " (3)	5.9
3. Normal pregnant, 2nd trimester	59	13.4 ± 3.5	(3) " (4)	<1
4. Normal pregnant, 3rd trimester	47	14.0 ± 4.7	(4) " (2)	3.0
5. Normal pregnant, 5 days post partum	53	22.3 ± 5.3	(4) " (5)	9.9
6. Hyperemesis gravidarum	17	10.3 ± 3.1	(2) " (6)	2.85

* Calculated,

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1}\frac{1}{n_2}}}$$

where $\bar{x}_1$ = average blood urea of one group of n_1 subjects, $\bar{x}_2$ = average blood urea of the other group of n_2 subjects, and s = average standard deviation of the two groups.

In view of the differences in fasting blood urea levels between the three groups of subjects a further study was made with larger groups of non-pregnant and normal pregnant persons. Table II gives the resultant data. It is again clear that blood urea was decreased during pregnancy; in several cases the change was apparent as early as the 6th week of gestation. Within a few days after the birth of the infant, blood urea rose to the normal non-pregnant level.

In Table III are shown the results of a test load of DL-alanine. A series of observations indicated that the maximum urea value was obtained at the 6th hour after alanine administration and that the blood level returned to normal within 12 hours in non-pregnant and normal pregnant subjects. For this reason the presentation of data was restricted to values prior to the test dose and those found at the 6th and 12th hours. Ranges of ure clearances are shown also; in all instances urea clearance was normal. I is obvious that patients with hyperemesis gravidarum responded differently to the test load of amino acid than did normal subjects, and the response became normal after the administration of pyridoxine. The normal sequence after the test load is a maximum value for blood urea at th 6th hour and a decrease to the original level by the 12th hour; the abnormal response is a failure to decrease between the 6th and 12th hours.

Table III

Effect of Test Load of Alanine on Blood Urea

Groups	No. of subjects	Mean blood urea at 0 hr.	Mean blood urea at 6th hr.	Mean blood urea at 12th hr.	Urea clearance range
		mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*
Normal females, non-pregnant	17	23.6	30.6	23.4	70–110
" pregnant, all trimesters	31	16.2	20.8	15.5	70–110
" " 5 days post-partum	15	24.0	29.4	24.2	90–140
Hyperemesis gravidarum on admission	14	10.8	16.2	15.1	75–100
After supportive therapy for 72 hrs.	8	13.8	16.6	16.9	80–110
" " " " 72 hrs., + 120 mg. pyridoxine	12	16.2	21.4	14.8	80–110

DISCUSSION

The investigation of Hawkins, MacFarland, and McHenry (10) showe that an increase in fasting blood urea accompanies the development o pyridoxine insufficiency in rats and that the blood urea response to a tes load of alanine is different from that obtained in normal rats. In dogs however, an elevation in blood urea was not observed, but there was a significant increase in urinary urea. Hawkins and Barsky (4) observed n change in blood or urinary urea in a human subject maintained on a lo pyridoxine diet for some weeks; the lack of any significant alteration migh indicate that a state of pyridoxine insufficiency was not obtained.

In the present study the administration of pyridoxine appeared to hav a significant effect upon fasting urea levels in the blood of patients showing marked nausea and vomiting in the first trimester of pregnancy, bu not in that of non-pregnant or normal pregnant women. There are a least two possible explanations of the observed effect. Regardless of th administration of pyridoxine, hospitalization and supportive therapy o the subjects with hyperemesis gravidarum may have made possible a better retention of ingested food with a consequent increased intake of pro tein and a resultant rise in blood urea; the supposed effect of pyridoxine might have been a coincidence. An obvious alternative explanation is that pyridoxine produced the observed alteration. The results obtained with a test load of an amino acid do not support the first explanation and definitely conform to the second.

The results reported above may be interpreted as presumptive evidence that in the cases of nausea and vomiting pyridoxine insufficiency was exhibited which was ameliorated by a supply of the vitamin. While the low level of fasting blood urea was opposite to the condition found in pyridoxine-deficient rats (10), the response to a test load of alanine was entirely similar. It might be assumed that the failure of pyridoxine to produce any effect in non-pregnant and normal pregnant subjects was due to the absence of a deficiency of the vitamin. We believe that the data may be most easily explained by the assumption that a pyridoxine insufficiency was present in the cases of hyperemesis gravidarum and that the results supply the first objective evidence of any effect of pyridoxine in humans. Recently Hobson (11) has observed, on the basis of an examination of food intakes, that pregnant women showing toxemia are possibly deficient in pyridoxine and niacin.

The employment of a test load of an amino acid was useful to detect a change in nitrogen metabolism, and the application of this procedure to other types of abnormalities would seem advantageous. Preliminary observations in cases of hepatitis strengthen this view.

It is useful to consider the results obtained recently on the protective action of pyridoxine against untoward effects resulting from deep x-ray treatment. An investigation on mice by Goldfeder *et al.* (12) showed that pyridoxine had a significant protective value. Two clinical reports (13, 14) have indicated that pyridoxine may have a similar effect in the reduction of nausea after irradiation to that which has been claimed in the nausea and vomiting of pregnancy. The question of whether irradiation induces a state of pyridoxine insufficiency is under investigation in our laboratory.

SUMMARY

In confirmation of the work of others, fasting blood urea was significantly less in normal pregnant than in non-pregnant subjects. The urea level was definitely lower in cases of hyperemesis gravidarum than in normal pregnancy, but was restored to a typical normal value after the administration of pyridoxine. Changes in blood urea after a test load of alanine were similar in normal pregnancy to those observed in non-pregnant persons, whereas subjects with hyperemesis gravidarum showed an abnormal response which was corrected after pyridoxine was given.

Thanks are expressed to the International Health Division of the Rockefeller Foundation for a grant to the Department of Public Health Nutrition which facilitated this investigation.

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LETTERS TO THE EDITORS

A METHOD FOR THE MICROBIOLOGICAL ASSAY OF VITAMIN B_{12}

Sirs:

It has been demonstrated that *Lactobacillus lactis* Dorner (ATCC 8000) must be cultivated with special precautions to prevent disassociation when used in the microbiological assay of vitamin B_{12}.[1] *Lactobacillus leichmannii* has been found to require nutrients similar to those needed by *Lactobacillus lactis* Dorner and to exhibit little tendency to disassociate.[2] The use of a stable test organism in the microbiological assay of vitamin B_{12} would be of distinct advantage.

The method herein outlined employs *Lactobacillus leichmannii* (ATCC 4797) as the test organism. The medium for the assay is a dehydrated product prepared by the Difco Laboratories, Inc., Detroit, Michigan, through the courtesy of Dr. C. W. Christensen.

The standard (purified vitamin B_{12} from Merck and Company, Inc.) and test materials are diluted to a concentration of 0.02 γ per ml. in distilled water. The test organism responds most satisfactorily within the range of 0.01 to 0.10 γ per tube levels. Duplicate or triplicate tubes on each level give growth response in good agreement turbidimetrically. Tubes containing only distilled water and the basal medium produce no growth in the absence of vitamin B_{12}. The results of the assay are evaluated turbidimetrically after 18 hours growth at 37°.

Inoculum for the test is prepared by washing four or five times in sterile saline the cells produced from a 24 hour growth of *Lactobacillus leichmannii* in Difco micro inoculum broth. Stab cultures are maintained on Difco stock culture agar for microbiological assays.

Additional work is in progress to determine the relative merits of this method in the assay of natural materials containing minute amounts

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*Basal Medium (Double Strength)**

	gm.		mg.
Bacto-vitamin-free casamino acids	12	Calcium-*d*-pantothenate	0.2
Dextrose	40	Folic acid	0.1
Sodium acetate	20	Biotin	0.0
L-Cystine, Difco	0.2	*p*-Aminobenzoic acid	0.2
DL-Tryptophan	0.2	Tween 80	200
Adenine	0.02	Dipotassium phosphate	1
Guanine	0.02	Monopotassium phosphate	1
			gm.
Uracil	0.02	Magnesium sulfate	0.4
	mg.		
Xanthine	1	Sodium chloride	0.02
Thiamine hydrochloride	2	Ferrous sulfate	0.02
Riboflavin	2	Manganese sulfate	0.02
			ml.
Niacin	2	Distilled water to make	1000
Pyridoxine hydrochloride	4		

The medium is adjusted to pH 6.8 before use.

* Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, **175**, 475 (194 Guirard, B. M., Snell, E. E., and Williams, R. J., *Arch. Biochem.*, **9**, 361 (194 Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, **174**, 1047 (1948). Shiv W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. A Chem. Soc.*, **70**, 2299 (1948). Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Che Soc.*, **70**, 2614 (1948).

of vitamin B_{12} compared with a similar assay in which *Lactobacillus lac* Dorner is the test organism.

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THE CONVERSION OF GLYCINE INTO SERINE IN THE INTACT RAT*

irs:

In an earlier communication[1] we have presented evidence indicating hat glycine is converted into serine via condensation with formate or a rmate derivative (Scheme I).

$$CH_2NH_2COOH + HCOOH \rightarrow CH_2OHCHNH_2COOH \qquad (I)$$

n the present investigation we have studied the physiological formation f this "formate." One possible mechanism is that glycine itself, by leamination[2] and subsequent decarboxylation, gives rise to "formate" Scheme II).

$$CH_2NH_2COOH \rightarrow CHOCOOH \rightarrow \text{"}HCOOH\text{"} + CO_2 \qquad (II)$$

We have investigated this pathway by degrading liver serine isolated fter the administration of glycine labeled with C^{14} in the methyl posiion. According to the proposed scheme the α- and β-carbon atoms of erine are both derived from the methyl carbon of glycine and should conain the isotope.

Four fasted rats weighing a total of 427 gm. were given 5 mM of glycine y stomach tube per 100 gm. After 14 hours the animals were sacriiced. Serine was isolated from the livers and degraded as previously lescribed.[1]

The results of the carbon analyses, shown in the table, are in accord vith Schemes I and II. The serine contained C^{14} in both the α- and 3-carbon atoms with almost as much activity in the β as in the α position.

The administered glycine contained 1.06 × 10^4 counts per minute per mg. of C the α-carbon.

	COOH*	α*	β*
erine	0	390	319

* Counts per minute per mg. of carbon.

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* Aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and by support of the Elisabeth Severance Prentiss Foundation.

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[2] Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, **152**, 119 (1944).

This experiment indicates that under certain conditions glycine itself is a major source of the formate or formate derivative for its conversion to serine.

The data of Winnick *et al.*[3] are in agreement with this hypothesis. Their serine isolated from liver homogenate equilibrated with C^{14}-methyl-labeled glycine contained a small amount of isotope in the β-carbon.

The author wishes to express his thanks to Dr. H. G. Wood for his interest in this investigation.

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[3] Winnick, T., Moring-Claesson, I., and Greenberg, D. M., *J. Biol. Chem.*, **175**, 127 (1948).

[4] With the technical assistance of Jean Lafaye.

THE EFFECT OF METHYLENE BLUE IN PREVENTING ALLOXAN DIABETES AND IN LOWERING THE BLOOD SUGAR OF ALLOXAN-DIABETIC RATS*

Sirs:

A number of compounds have been found to prevent the diabetic action of alloxan when administered immediately before the alloxan.[1]

The writers have observed that the oxidation-reduction dye, methylene blue, not only is protective against the diabetogenic action of alloxan when given before the alloxan, but also exerts a marked blood sugar-lowering effect when administered to rats previously made diabetic with alloxan. This paper records the blood sugar-lowering effect on twenty-two animals. Experiments to be published in more detail later will confirm the validity of this preliminary report.

The following experiments illustrate the effect of methylene blue (MB) in preventing alloxan diabetes.

Five non-fasted rats (240 to 267 gm.) were given 1 cc. of 1 per cent MB[2] 30 minutes prior to the injection of 150 mg. of alloxan per kilo of body weight. 24 hours later the blood sugar levels ranged from 28 to 124 mg. per cent (average 59 mg. per cent). Four non-fasted controls treated similarly but without MB showed 24 hour blood sugars of 113 to 299 mg. per cent (average 200 mg. per cent).

When six rats (189 to 310 gm.) were fasted for 48 hours and then treated with MB and alloxan as above, the blood sugars after 48 hours ranged from 0.0 to 90 mg. per cent (average 60 mg. per cent). Five 48 hour-fasted controls (187 to 232 gm.) treated with alloxan but not with MB showed 48 hour blood sugars ranging from 119 to 650 mg. per cent (average 397 mg. per cent).

The effect of MB in lowering the blood sugar of alloxan-diabetic rats is shown in the table. The dye was injected into non-fasting rats, in some cases 2 days and in others 1 to 3 months after alloxan injection.

In our experience MB in the dosage used does not alter the blood sugar of normal animals but causes definite hypoglycemia in rats recently made diabetic with alloxan.

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* Aided by a grant from the Diabetic Research Foundation of Portland, Oregon.

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[2] Aqueous solutions of methylene blue and alloxan monohydrate (freshly prepared) were injected intraperitoneally. Animals were given nembutal prior to injection or blood sampling.

Effect of Methylene Blue in Lowering Blood Sugar of Non-Fasting Alloxan-Diabetic Rats*

Animal No.	Blood sugar, mg. per cent									
	2 days after alloxan	Days after methylene blue								
		2	4	8	9	15	17	24	49	51
1	295	72	45		61		73	119		
2	243	79	147		51		147			
3	350	96	51		73		68	102		102
4	457	73	328			73				
5	664	51	565			130		424	277	
6	689	355	486			203		339	345	
7	407†	232		327			379			
8	528†	45								
9	382†	102		237			266			
10	301†	102		170			220		119	
11	492†	277								

* The dose of methylene blue was 1 cc. of 1 per cent solution in all experiments and was given immediately after withdrawal of blood used to determine the effect of alloxan injection 2 days previously.

† Chronically diabetic rats; diabetes of 1 to 3 months duration.

Studies relating to the mechanisms of the protective and blood sugar-lowering effects of methylene blue in alloxan diabetes, and of the possible effect of methylene blue in pancreatic diabetes, are in progress.

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SEPARATION OF THE GROWTH FACTORS FOR LEUCONOSTOC CITROVORUM AND LACTOBACILLUS LEICHMANNII BY MEANS OF ELECTROLYSIS

Sirs:

The activity of a crystalline preparation of the antipernicious anemia factor for *Lactobacillus leichmannii* 313 has been reported by Hoffmann and coworkers.[1] Skeggs *et al.*[2] used *Lactobacillus leichmannii* 4797 as an assay organism in their studies on the isolation of the animal protein factor. The growth factor for *Leuconostoc citrovorum* 8081 has been studied by Sauberlich and Baumann,[3] who found that liver extracts prepared for the treatment of pernicious anemia were also concentrated sources of the growth factor for this organism.

Electrolysis cell No.	Final pH of cell content	Titration values for bacterial growth factor tests,* 0.1 N NaOH		
		Leuconostoc citrovorum 8081	*Lactobacillus leichmannii* 4797	*Lactobacillus leichmannii* 313
		ml.	*ml.*	*ml.*
1†	5.4	0.23	4.62	4.25
2	3.6	0.26	2.66	2.26
3	3.4	0.34	0.82	0.62
4	3.3	0.67	0.67	0.54
5	3.2	2.63	0.60	0.62
6‡	3.0	4.39	0.78	0.56
Blank		0.24	0.62	0.57

* 5 ml. aliquots from 10 ml. cultures, 72 hour growth periods.

† Negative electrode in this cell.

‡ Positive electrode in this cell.

During a comparative study of the factors for these three organisms by the present authors, the *citrovorum* factor was separated from the other factors by electrolysis of a liver extract concentrate (reticulogen) in a specially designed six cell apparatus. In 0.02 M acetic acid the activity for both of the *leichmannii* strains migrated toward the negative electrode, while the activity for *Leuconostoc citrovorum* migrated toward the positive electrode as indicated in the table.

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At the start of the experiment, 0.5 ml. of reticulogen in 0.02 M acetic acid was placed in Cell 4. The rest of the apparatus (total volume 280 ml.) was filled with 0.02 M acetic acid. After the electrolysis, one 125th part of the contents of each cell was used in the tests with *Leuconostoc citrovorum* and one 6250th part from each cell was used in the tests with both *Leichmannii* strains. Part of the activity for *Leuconostoc citrovorum* was lost during the electrolysis, presumably by oxidation at the positive electrode.

These results indicate that the *citrovorum* and *leichmannii* factors are not identical, that the *leichmannii* factor contains a basic group, and that the *citrovorum* factor contains a fairly strong acidic group. When electrolyzed in 0.05 M NH_4OH, the activity for all three organisms migrated toward the positive electrode, indicating the presence of a weaker acidic group in the *leichmannii* factor.

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DIFFERENCE IN GROWTH-PROMOTING EFFECT OF DESOXYRIBOSIDES AND VITAMIN B_{12} ON THREE STRAINS OF LACTIC ACID BACTERIA[*]

Sirs:

Snell *et al.*[1] recently showed that thymidine is a growth factor for some lactic acid bacteria. Shive *et al.*[2] and Wright *et al.*[3] found that for *L. lactis* Dorner thymidine is able to replace the requirement for a liver concentrate (vitamin B_{12}).

Growth-Promoting Effect of Desoxyribosides and Vitamin B_{12} Concentrates

Addition per 10 ml. medium[*]	Galvanometer reading[†]		
	Tbm. lactis 1, 20 hrs., 37°	*Tbm. jugurt* 13, 40 hrs., 37°	*Tbm acidophilus* R26, 20 hrs., 37°
0, inoculated	85	96	94
5 γ thymidine[‡]	42	57	25
5 " guanine desoxyriboside[§]	48	58	27
50 " thymine	92	95	94
0.5 μl. liver concentrate[‖]	33	48	94
0.1 mμgm. B_{12} concentrate[¶]	31	62	94
1 γ folic acid	83	96	93
25 mg. tryptic casein digest	56	95	95

[*] A previously described medium[4] which contains all of the known nutritional essentials for lactic acid bacteria was used in the following modification: Difco-yeast extract was omitted; 100 mg. of lactose and 1 mg. of sodium pyruvate were added per 10 ml. The tryptic casein digest was replaced by 50 mg. of acid-hydrolyzed casein and 5 mg. of tryptic casein digest per 10 ml.

[†] Per cent incident light transmitted.

[‡] We are indebted to Dr. W. Shive and Dr. R. Hotchkiss for samples of thymidine.

[§] Prepared by Dr. M. Friedkin (*cf.* Friedkin, M., Kalckar, H. M., and Hoff-Jørgensen, E., *J. Biol. Chem.*, **178**, 527 (1949).

[‖] A commercial antipernicious liver extract from the Danish firm Gea 1 ml. ~ 50 gm. of liver. 100 ml. contain about 15 mg. of dry matter.

[¶] We are indebted to Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, for a sample of this concentrate.

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[*] This work has been supported by a grant from the Rockefeller Foundation.

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In the course of a survey of the nutritional requirements of certain lactic acid bacteria of the genus *Thermobacterium*[5] a strain, *Tbm. acidophilus* R26, was encountered whose requirement for thymidine could not be replaced by either a B_{12} concentrate or a commercial liver preparation. For another strain, *Tbm. lactis* 1, the effect of thymidine or vitamin B_{12} may be replaced by charcoal-treated tryptic casein digest (see the table). The third strain, *Tbm. juhurt* 13, grows on either thymidine or vitamin B_{12}, but tryptic casein digest is without any effect.

Guanine desoxyriboside has the same effect on all three strains as thymidine.

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[5] We are indebted to Professor S. Orla-Jensen and to Mrs. Snog-Kjaer for cultures of these organisms from the Orla-Jensen collection.

ENZYMATIC SYNTHESIS OF DESOXYRIBOSE NUCLEOSIDE WITH DESOXYRIBOSE PHOSPHATE ESTER*

Sirs:

Recent observations[1] indicate that desoxyribose nucleosides can be split by an enzymatic phosphorolysis analogous to that described for ribonucleosides.[2] We wish to report the isolation of the primary phosphorolysis product, an extremely acid-labile desoxyribose phosphate ester (DRP) which can react with hypoxanthine to form hypoxanthine desoxyriboside.

The Warburg vessel contained guanine desoxyriboside (0.0123 M);* alanylglycine, pH 8.55 (0.082 M); Na_2HPO_4 (0.143 M); 12 mg. of nucleoside phosphorylase; 2.1 mg. of xanthine oxidase; and 0.25 mg. of catalase in a total volume of 2.44 ml. Incubated in air at 13° for 27 hours, oxygen uptake, 15.5 μM. DRP (see the text) was then incubated with hypoxanthine, as indicated below.

Experimental conditions		Desoxyriboside synthesized	
		Differential spectrophotometric analysis	Microbiological assay
		μM	μM†
Complete system contained 0.12 μM hypoxanthine; DRP, 1 μM labile P: alanylglycine, pH 8.55 (0.05 M); and 0.5 mg. nucleoside phosphorylase in total volume of 1.2 ml. Incubated at 30° for 30 min.	Complete system	0.067	0.087
	DRP not added during incubation but after inactivation of enzyme	0	0.008

* The values in parentheses refer to the final concentration in the complete mixture.

† Expressed as micromoles of thymidine which evoke an equal growth response.

Guanine desoxyriboside, prepared in crystalline form from thymus nucleic acid, was incubated with liver nucleoside phosphorylase[2] as indicated in the table. The free guanine was oxidized to uric acid in the presence of xanthine oxidase. After removal of the uric acid by centrifugation, the incubation mixture was extracted with *n*-butanol and diethyl ether to denature the protein, and then freed of residual purines by treatment with norit. The clear filtrate, after removal of inorganic phosphate as $MgNH_4PO_4$, was evaporated to a small volume and used for experiments in synthesis, as shown in the table.

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* This work has been supported by grants from the Donner Foundation, Inc., the Lederle Laboratories Division, American Cyanamid Company, the Rockefeller Foundation, and the Carlsberg Foundation.

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The phosphate ester thus obtained releases both inorganic phosphate and desoxyribose upon very mild hydrolysis (50 per cent splitting in 12 minutes at 25° in pH 4 acetate buffer). Labile phosphate is determined as $MgNH_4PO_4$, since the high instability of DRP at pH 4 excludes the use of the Lowry-Lopez method.[3] A yield of 18 μM of DRP (expressed as labile P) was obtained from 30 μM of guanine desoxyriboside.

The ester is most specifically characterized by its ability in the presence of liver nucleoside phosphorylase to exchange its phosphate for hypoxanthine. The formation of hypoxanthine desoxyriboside was demonstrated by differential enzymatic spectrophotometry[2] and further substantiated by the ability of the enzymatically produced desoxyriboside to replace thymidine as a growth factor for *Thermobacterium acidophilus* R26.[4] On a molar basis the stimulatory effect of the synthesized desoxyriboside was approximately equal to that of thymidine or guanine desoxyriboside.

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[3] Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).

[4] Hoff-Jørgensen, E., *J. Biol. Chem.*, **178**, 525 (1949).

[5] National Institute of Health Fellow.

THE UTILIZATION OF THE α-CARBON ATOM OF GLYCINE FOR THE FORMATION OF ACETIC AND ASPARTIC ACIDS*

Sirs:

We have been able to demonstrate that in the rat acetic and aspartic acids can be formed from glycine. Glycine labeled with C^{14} in the methylene group was fed to a 250 gm. rat at a level of 80 mg. per day together with 250 mg. of *l*-α-amino-γ-phenylbutyric acid for 3 days. The excreted acetyl-*l*-aminophenylbutyric acid, isolated from the urine, was hydrolyzed and the resulting acetic acid obtained as the silver salt. The silver acetate was degraded with bromine in carbon tetrachloride[1] and the resulting CO_2 precipitated as $BaCO_3$. Aspartic acid was isolated from the proteins of the internal organs and decarboxylated by ninhydrin. Urinary urea was isolated as the dixanthydryl derivative. The urea carbon is a representative sample of the respiratory CO_2.[2] The results are summarized in the table.

Compound	Activity*
Glycine fed	1,370,000
Dixanthydryl urea (respiratory CO_2)	2,600
Silver acetate from acetylaminophenylbutyric acid	4,200†
$BaCO_3$ from carboxyl carbon of acetic acid	4,300†
Acetic acid (α-carbon)	4,100‡
Aspartic acid	1,300
$BaCO_3$ from carboxyls of aspartic acid	700†
Aspartic acid (α- and β-carbon atoms)	1,800‡

* Counts per minute per standard dish (2.41 sq. cm.) of an "infinitely thick" sample of carbon.
† Corrected for back-scattering.
‡ Value calculated from the data.

The data demonstrate that both carbon atoms of the acetic acid were derived from the α-carbon atom of glycine. The activity of the α- and β-carbon atoms of the aspartic acid is 2.5 times that of its carboxyl groups. The latter could derive their activity from the respiratory CO_2. This distribution of C^{14} in the aspartic acid is consistent with the view that oxalacetic acid is the source of α,β-labeled pyruvic acid, which could acetylate aminophenylbutyric acid either directly or following conversion

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*This work was supported by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

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to acetic acid.[3] That the α- and β-carbon atoms of aspartic acid should be less active than those of acetic acid is to be expected, since not all of the aspartic acid of the tissue proteins has been regenerated in the 3 day period.

A hypothetical mechanism for these reactions can be deduced from the well known condensation of derivatives of glycine with carbonyl compounds. It is possible that glyoxylic acid, derived from glycine by deamination,[4] condenses with glycine to form a 4-carbon compound[5] labeled in the α and β positions, which is in equilibrium with aspartic acid.

An alternative mechanism is the degradation of the glycine to labeled formic acid and condensation of the latter with glycine to α,β-labeled serine.[6] Pyruvic acid derived from serine[7] would then be the precursor of the acetic and oxalacetic acids. Further investigations are required in order to decide this question.

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EVIDENCE OF THE EXISTENCE OF A CORE IN DESOXYRIBONUCLEIC ACIDS

Sirs:

The action of crystalline desoxyribonuclease[1] (supplied by the Worthington Biochemical Laboratory, Freehold, New Jersey) on the desoxyribonucleic acids of calf thymus and of yeast[2] leads to the production of dialyzable fragments, without liberation of NH_3 or inorganic P. But even after prolonged digestion a non-diffusible core remains.

	Calf thymus, Experiment 1			Calf thymus, Experiment 2	Yeast		
	Original	Dialysate	Dialysis residue	Dialysis residue	Original	Dialysate	Dialysis residue
Digestion, *hrs*	0	6	24	72	0	6	24
Dialysis, *hrs*	0	6	72	158	0	6	24
Distribution of fractions, % of *original*	100	53	7	1.4	100	58	5
	Composition of fractions, molar proportions						
$\frac{\text{Adenine}}{\text{Guanine}}$	1.2	1.2	1.6		1.7	1.5	
$\frac{\text{Thymine}}{\text{Cytosine}}$	1.3	1.2	2.2				
$\frac{\text{Adenine}}{\text{Cytosine}}$	1.6	1.2	3.8				
$\frac{\text{Purines}}{\text{Pyrimidines}}$	1.2	1.0	2.0				

Both the dialyzable portion (6 hours digestion) and the core remaining after additional digestion and dialysis were analyzed[3] for purines and pyrimidines. Whereas the composition of the former did not differ appreciably from that of the original highly polymerized nucleic acids[4, 5] (except a change in the adenine to cytosine ratio), the core studied in the case of the thymus nucleic acid exhibited a significant divergence. The data provided in the accompanying table indicate a considerable increase in the molar proportions of adenine to guanine and especially to cytosine, of thymine to cytosine, and of purines to pyrimidines. Thus, the dissymmetry in the distribution of constituents, found in the original nucleic acid,[4] is intensified in the core.

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Apart from less probable explanations, these findings could be interpreted as indicating either the presence in the nucleic acid sample of a small quantity of a second desoxypentose nucleic acid of different purine and pyrimidine composition or the existence in the desoxyribonucleic acid chain of nucleotide clusters (in the case studied, relatively richer in adenine and thymine) distinguished from the bulk of the molecule by greater resistance to enzymatic disintegration.

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RELEASE OF FREE AND BOUND FORMS OF BIOTIN FROM PROTEINS*

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(Received for publication, September 30, 1948)

The existence of biotin in tissues in a strongly bound form was early reported by Snell, Eakin, and Williams (1). These workers found that autolysis increased the amount of biotin that could be extracted from liver. Other workers including Cheldelin *et al.* (2), Thompson, Eakin, and Williams (3), and Lampen, Bahler, and Peterson (4) found that acid or enzymatic hydrolysis gave maximum yields of biotin from a variety of natural materials. In 1944 Wright and Skeggs (5) reported that biotin values obtained with the *Lactobacillus casei* method of Landy and Dicken (6) were higher than those obtained with *Lactobacillus arabinosus*. When the substances were hydrolyzed further with acid, the biotin content as determined with *Lactobacillus arabinosus* increased to the values previously obtained with *Lactobacillus casei*. These data suggest that certain combined forms of biotin are available to *Lactobacillus casei* but not to *Lactobacillus arabinosus*.

The present investigation was undertaken to obtain information regarding the release of biotin from proteins by acids and enzymes and to determine how much of this soluble biotin was free and how much of it was combined. It was hoped that the data thus obtained would throw some light on the type of linkage binding biotin to protein.

EXPERIMENTAL

Free and Bound Biotin in Protein Hydrolysates

Three microbiological assays were used for the determination of soluble biotin: the *Lactobacillus casei* method of Shull, Hutchings, and Peterson (7), the *Lactobacillus arabinosus* method of Wright and Skeggs (5), and the *Saccharomyces cerevisiae* assay of Hertz (8). Several 1 gm. samples (dry basis) of fresh untreated beef liver, commercial casein, and fibrin were hydrolyzed, each with 10 ml. of sulfuric acid varying in normality from 2 to 18, at 120° and assayed for biotin by all three methods. The values for each of the samples as well as for a 1 γ sample of synthetic biotin are presented in Table I. The values obtained with *Lactobacillus arabinosus* were generally lower than those obtained with *Lactobacillus casei* or *Saccharomyces cerevisiae.* Even when the concentration of the acid was increased to 18 N, no better agreement among the assays was reached. The 18 N sulfuric acid was sufficient, however, to destroy about three-fourths of the synthetic biotin tested as a control.

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The difference in response of the three microorganisms may be due to the liberation of some complex during hydrolysis which had biotin activity for *Lactobacillus casei* and *Saccharomyces cerevisiae* but not for *Lactobacillus arabinosus.* A second explanation might be the partial destruction of the biotin molecule during acid hydrolysis, rendering it unavailable to *Lactobacillus arabinosus* but without causing it to lose activity for the other two organisms. A third explanation is that *Lactobacillus casei* and *Saccharomyces cerevisiae* were responding to some compound other than biotin.

TABLE I

*Biotin Activity of Acid-Hydrolyzed Liver, Fibrin, Casein, and Synthetic Biotin**

Sample	H_2SO_4 concentration	Biotin activity per gm. (dry basis)		
		L. casei	*L. arabinosus*	*S. cerevisiae*
	N	*mμgm.*	*mμgm.*	*mμgm.*
Beef liver	4	3270	2490	3740
" "	9	4450	3280	4650
" "	18	4340	2500	4350
Fibrin	2	265	135	307
"	4	302	215	450
"	9	360	250	328
Casein	2	52	15	45
"	4	108	25	65
"	9	70	30	63
Synthetic biotin (1 γ)	4	815	932	975
" " (1 ")	9	620	755	745
" " (1 ")	18	175	238	225

* Hydrolysis, 2 hours at 120°.

A Craig type extraction procedure was employed for separation of the two kinds of active compounds. The apparatus consisted of ten separatory funnels mounted in a series on a vertical rod resting on a spring base so that the rod and funnels could be bounced up and down to bring about mixing of the liquids contained in the separatory funnels. The solvents used in the extraction were *n*-butanol and water, with water as the moving phase. 10 ml. aliquots of each solvent previously saturated with the other were used throughout the extraction, which was carried out at 25° and pH 1. After the extraction the biotin content of the water layer of each cell was determined. Fig. 1, Curve 1, shows the distribution of synthetic biotin after such an extraction. The maximum biotin concentration appeared at Cells (funnels) 2 and 3. Curves 2 and 3 (Fig. 1) give the distribution of biotin-active compounds in 1 gm. of liver (dry) which had been hydrolyzed for 1 hour at 100° with 10 ml. of 4 N sulfuric acid. When the samples from each of the cells after extraction were assayed with *Lactobacillus casei*, the existence of at least two biotin-active components was indicated. One component appeared in maximum concentration at Cells 2 and 3, corresponding to the maximum obtained with synthetic biotin. The other appeared in highest concentrations in Cells 8 and 9. When *Lactobacillus arabinosus* was used to assay the samples, only one maximum appeared, that at Cells 2 and 3. When samples taken from each of the cells were hydrolyzed further with 4 N sulfuric acid for 1 hour at 120° and reassayed, two maxima were observed with each organism (Curves 4 and

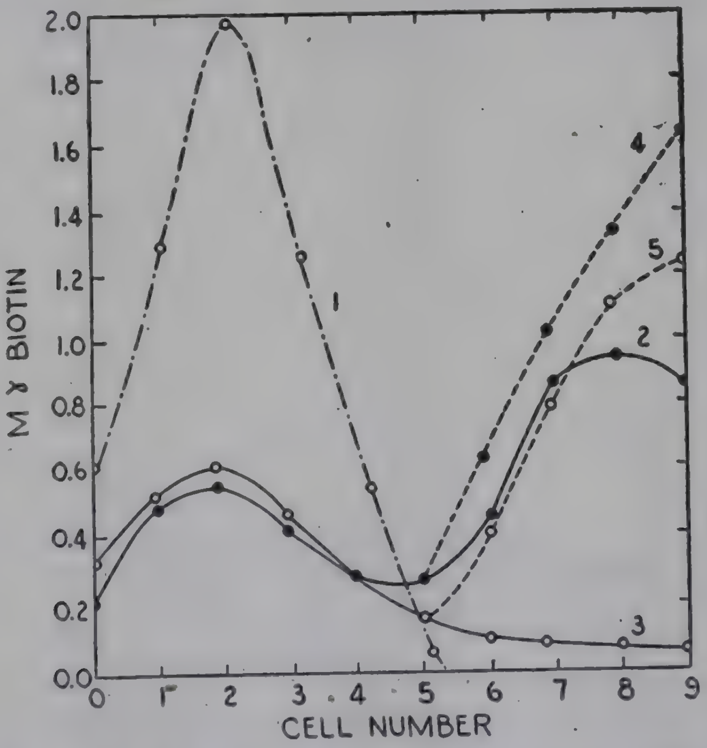

FIG. 1. Separation of the forms of biotin in acid-hydrolyzed liver by Craig extraction. Curve 1, distribution of synthetic biotin; Curve 2, biotin activity of acid-hydrolyzed liver by *Lactobacillus casei* assay; Curve 3, biotin activity of liver hydrolysate by *Lactobacillus arabinosus* assay; Curve 4, biotin activity of liver hydrolysate after second hydrolysis by *Lactobacillus casei* assay; Curve 5, same sample as in Curve 4, by *Lactobacillus arabinosus* assay.

5, Fig. 1). From the data it appears that the component possessing biotin activity for *Lactobacillus casei* but not for *Lactobacillus arabinosus* may have been a bound form of biotin unavailable to *Lactobacillus arabinosus* until hydrolyzed further.

Because the biotin which was active for *Lactobacillus arabinosus* was similar to synthetic biotin in solubility as measured by the counter-current extraction, it was decided to call this free biotin, although it was realized that there was not sufficient evidence for assuming that this biotin was unattached molecular biotin. The soluble biotin available to *Lactobacillus casei* but not to *Lactobacillus arabinosus* was called bound biotin.

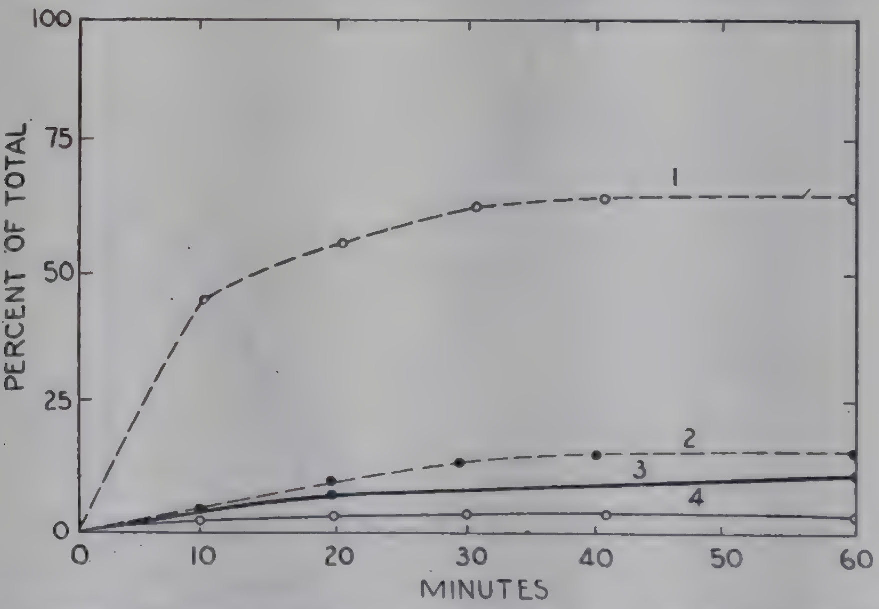

FIG. 2. Release of nitrogen and biotin by acid hydrolysis of liver. Curve 1, total soluble nitrogen; Curve 2, soluble free amino nitrogen; Curve 3, free biotin; Curve 4, ammonia nitrogen. Nitrogen content of liver 11.0 per cent, biotin content of liver 3.65 γ per gm., dry weight.

Release of Forms of Nitrogen and Biotin by Acid and Enzymatic Hydrolysis

10 gm. samples of fresh untreated liver, commercial casein, and fibrin were hydrolyzed in 100 ml. of 4 N sulfuric acid at 100° for various lengths of time. The solutions were filtered and analyzed for biotin by the *Lactobacillus casei* and *Lactobacillus arabinosus* methods, for total soluble nitrogen by the micro-Kjeldahl method of Johnson (9), for amino nitrogen by formol titration (10), and for ammonia nitrogen by aeration (11). The results were calculated as percentage of total nitrogen or total biotin based on the original sample. The results obtained on the liver hydrolysate are presented in Fig. 2. The linkages binding the amino nitrogen, biotin, and total nitrogen have almost the same stability to acid, as is shown by the similarity of the slopes of these three curves after the first 10 minutes of

hydrolysis. The initial differences in the slopes may be partially explained by the presence of appreciable amounts of soluble biotin or nitrogen in the liver before hydrolysis.

The acid hydrolysis curves for casein and fibrin were similar to those for liver, except that with casein over 60 per cent of the total biotin was released in 60 minutes instead of only 11 per cent as in the case of liver. Bound biotin released from casein and fibrin by acid was less than 5 per cent of the total, while with liver the bound biotin released was equal to the free biotin.

Enzymatic hydrolyses were also carried out on samples of fresh liver. Trypsin and papain were used in concentrations of 1 gm. per 100 ml. of liver suspension containing 10 gm. of liver (dry basis). The pH of the hydrolysis mixture was buffered at 7.8 for trypsin and 5.0 for papain. Samples were taken at various intervals up to 60 hours, heated to boiling to inactivate the enzymes, and analyzed for nitrogen and biotin. The data for liver hydrolyzed with papain are given in Fig. 3. The data for the trypsin hydrolysis were very similar to the papain data. Almost all of the biotin was liberated by the enzymes during the early stages of hydrolysis, while soluble nitrogen amounted to only 50 to 60 per cent of the total nitrogen in the liver in the same length of time. This is in marked contrast to the acid hydrolysis of liver in which the free biotin did not rise above 11 per cent. A possible explanation for the early rapid liberation

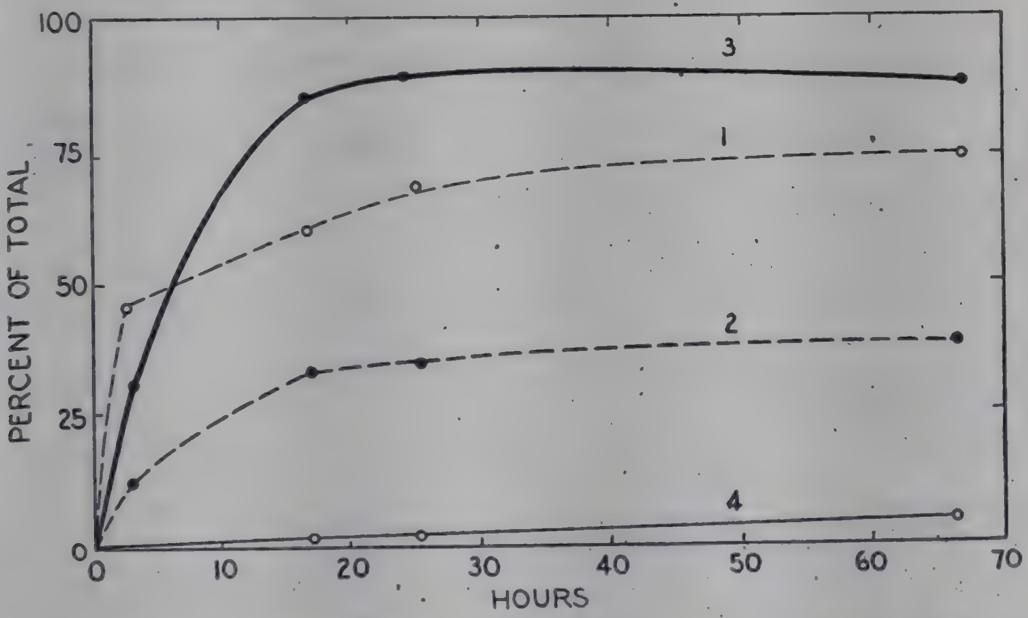

FIG. 3. Release of nitrogen and biotin from liver by hydrolysis with papain. Curve 1, total soluble nitrogen; Curve 2, soluble free amino nitrogen; Curve 3, free biotin; Curve 4, ammonia nitrogen. Nitrogen content of liver 11.3 per cent, biotin content 4.0 γ per gm., dry weight.

of free biotin is that the biotin molecules may be released from the end o peptide chains, at which positions they are most rapidly attacked by peptidases. Other types of enzymes such as esterases or phosphatases may possibly be responsible for the release of biotin.

Table II gives the amount of free and soluble bound biotin liberated from 1 gm. samples of liver hydrolyzed for 1 hour with 0.1 gm. of papain (Merck) and trypsin (Difco). The amounts of free and soluble bound biotin were determined by separating them in the counter-current extraction apparatus and assaying with *Lactobacillus casei*. It can be seen from Table II that the amount of biotin liberated in 1 hour represented only about 2 per cent of the total (6.0 γ per gm., dry weight). The biotin liberated in 24 hours was equivalent to the total present in liver, and all of this biotin was in the free form.

TABLE II

Release of Free and Soluble Bound Biotin from Liver by Enzymes

Commercial sample	Time of hydrolysis	Free biotin per gm. of dry matter	Soluble bound biotin per gm. of dry matter
	hrs.	γ	γ
Trypsin	1	0.038	0.042
“	24	5.48	<0.01
Papain	1	0.076	0.070
“	24	6.10	<0.01
Crystalline enzymes			
Pepsin	1	0.093	0.080
“	24	0.093	1.74
Chymotrypsin	1	0.113	<0.01
“	24	0.126	0.074
Papain	1	0.090	0.030
“	24	0.103	0.247

The liberation of biotin by enzymes was further studied by observing the action of crystalline enzymes on liver which had been autoclaved for 10 minutes to inactivate the naturally occurring enzymes. 10 mg. of pepsin, chymotrypsin, or papain (this enzyme was purified but not crystalline) were added to 100 ml. of buffered liver suspension containing 3 gm. of liver (dry weight), and the mixture was allowed to hydrolyze at 37° for 48 hours. The pepsin digestion mixture was buffered with 0.1 M phosphoric acid at pH 1.5, the chymotrypsin with 0.1 M potassium phosphate at pH 7.5, and the papain hydrolysis mixture with 0.1 M sodium citrate at pH 5.0. The papain mixture was also activated by the addition of 1 ml. of 0.01 M KCN. 10 ml. aliquots were removed at various intervals, heated in a boiling water bath for 15 minutes, and analyzed for free and total biotin, free amino nitrogen, and total soluble nitrogen. The data for the liberation of free and soluble bound biotin are included in Table II. In Fig. 4 is graphically represented the liberation of nitrogen and biotin during the pepsin hydrolysis. The data show that although an appreciable amount of the total nitrogen was solubilized the amount of free biotin liberated was low. Pepsin liberated 68 per cent of the total biotin and 85 per cent of this (58 per cent of the total) was in the bound form. Chymotrypsin was much less effective, releasing only about 8 per cent of the total and of this about 60 per cent was free biotin. The figures for papain were 4 per cent of the total released as free biotin and 12 per cent as bound biotin. Because of the high amount of bound biotin liberated during the pepsin hydrolysis, this enzyme is an effective agent for preparing a solution of bound biotin for further purification.

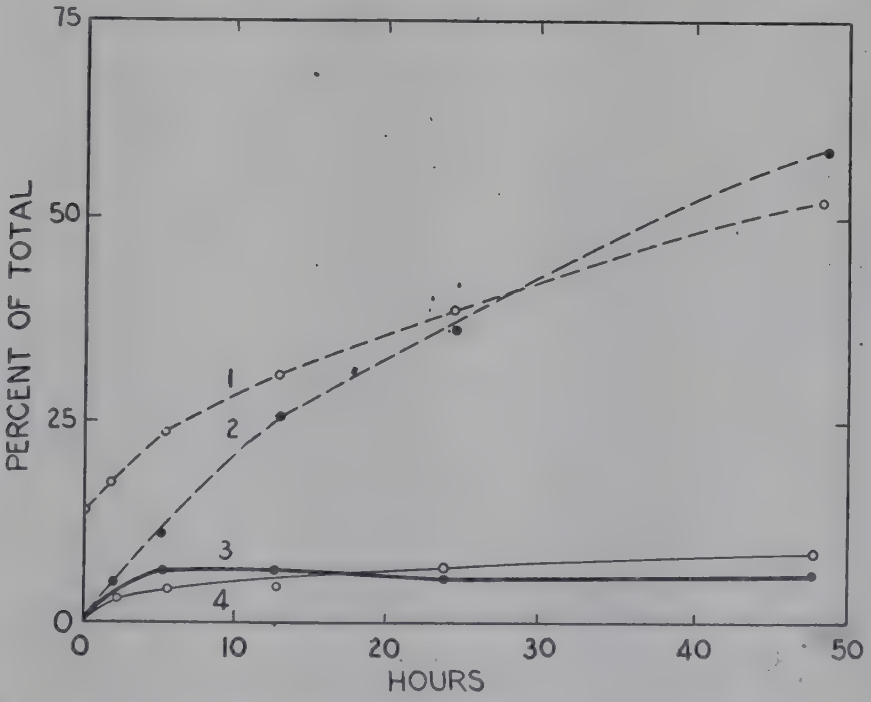

Fig. 4. Release of nitrogen and biotin from liver by crystalline pepsin. Curve 1, total soluble nitrogen; Curve 2, soluble bound biotin; Curve 3, free biotin; Curve 4, soluble free amino nitrogen.

Resolution of Bound Biotin Components by Paper Chromatography

A 3 gm. sample of liver was hydrolyzed with 10 ml. of 4 N sulfuric acid at 100° for 1 hour and subjected to counter-current extraction. The bound biotin contained in Cell 9 of the apparatus was evaporated *in vacuo* to a concentration of 40 to 50 mμgm. per ml. The solution was next subjected to chromatographic resolution of the biotin-active components based on the paper chromatographic method described by Consden *et al.* (12).

A paper column 1 to 4 cm. by 40 cm. was used, and on it a 0.03 ml. sample in water solution was streaked, about 5 cm. from the top, and allowed to dry. The column was then suspended for several hours in the glass jar, to enable it to absorb water vapor from the atmosphere of the jar, which was kept saturated with water vapor and butanol by placing a beaker of each of these liquids in the jar. The top of the column was immersed in a beaker of water-saturated butanol to a depth of 3 cm. The apparatus was then allowed to stand until 2 to 4 ml. of butanol had passed down the column (20 to 40 hours), after which the column was dried and cut up into 1 cm. squares. Each of these squares was placed in a *Lactobacillus casei* assay tube and the biotin activity determined after 3 days by titration of the acid produced.

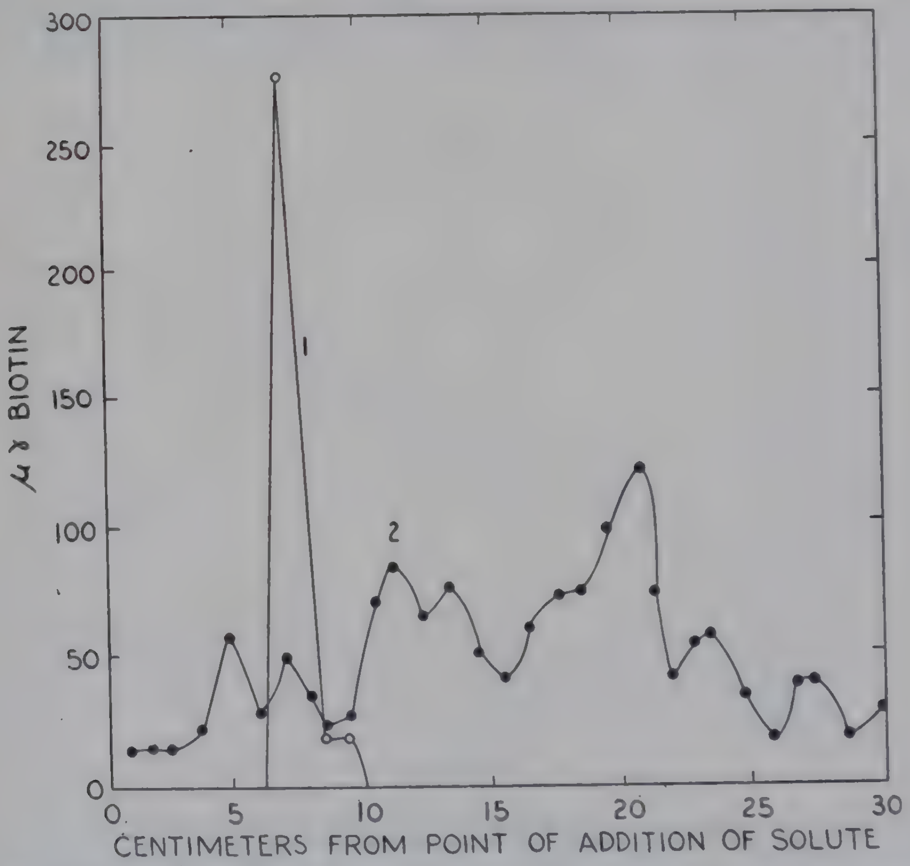

Fig. 5. Resolution of biotin components in acid- and pepsin-digested liver by paper chromatography. Curve 1, liver digested with crystalline pepsin; Curve 2, acid-hydrolyzed liver.

Another column containing bound biotin prepared by digestion of liver with pepsin was developed and assayed. The bound biotin solution added to the column was prepared by hydrolyzing 30 gm. of autoclaved liver with 30 mg. of crystalline pepsin at 37°, pH 1.5. The hydrolysate was filtered and the solution evaporated *in vacuo* to 10 ml. The distribution of the biotin-active components for both columns after 30 hours is given in Fig. 5. The chromatogram for the acid-hydrolyzed liver sample contains many components, as would a chromatogram of any acid-protein hydrolysate in which a given amino acid is located at various places on the column according to its attachment to peptides of various lengths. Essentially one component was observed when the crystalline pepsin liver digest was chromatographed. If, as indicated by the chromatogram, the soluble bound biotin is mainly one component, then chromatography on a larger scale may be used as a method for purifying and isolating bound biotin.

DISCUSSION

The ease with which free biotin is liberated from liver by hydrolysis with commercial proteolytic enzyme preparations indicates that biotin may be attached to the protein by peptide linkages. However, since the true nature of the linkage is not known, other types of enzymes ordinarily found in enzyme preparations (phosphatases, esterases, etc.) may be involved in the reaction. The rapid liberation of the biotin during the early stages of the hydrolysis is analogous to the rapid liberation of arginine during the enzymatic digestion of proteins (13).

The liberation by crystalline pepsin of over 50 per cent of the total biotin from liver as soluble bound biotin which could not be resolved into more than one major component on the chromatographic column indicates that only one type of biotin complex exists in the protein molecule. The release of a single major component containing biotin would be expected to occur when the protein is hydrolyzed by a single enzyme, specific in its point of attack, and the reaction is allowed to proceed until no further hydrolysis takes place.

SUMMARY

Samples of casein, fibrin, and liver hydrolyzed by sulfuric acid or enzymes were found to have biotin activities for the assay organism *Lactobacillus arabinosus* that differed from their activities for *Lactobacillus casei* and *Saccharomyces cerevisiae*. This was believed to be due to the presence of some of the biotin in a bound or unavailable form. By means of a Craig type extraction, a separation of the biotin-active components into two fractions was effected. One component was equally active for *Lactobacillus arabinosus* and *Lactobacillus casei* and showed solubility properties similar to those of synthetic biotin on counter-current extraction. The second component was not active for *Lactobacillus arabinosus*, but possessed activity for the other two organisms. The component inactive for *Lacto-*

bacillus arabinosus could be rendered available to that organism by further hydrolysis with either acids or enzymes.

Biotin was freed less rapidly than total nitrogen during acid hydrolysis. When commercial preparations of trypsin, papain, or other enzymes were used as hydrolyzing agents, the biotin was released more rapidly than the nitrogen and was almost completely liberated after 24 hours. Crystalline proteinases liberated very little free biotin from liver. Pepsin released almost 60 per cent of the total biotin as soluble bound biotin in 48 hours.

The soluble bound biotin from acid-hydrolyzed liver samples was resolved into several components on a paper chromatographic column, while soluble bound biotin liberated from liver by crystalline pepsin appeared to be mainly one compound.

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STUDIES ON THE HEAT INACTIVATION OF METHIONINE IN SOY BEAN OIL MEAL*

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(Received for publication, October 4, 1948)

Previous work has demonstrated that autoclaving soy bean oil meal for 1 hour at 25 pounds pressure, or for 4 hours at 15 pounds pressure, leads to a decrease in the nutritive quality of the protein as measured by its effect on the growth of chicks (1–4). Furthermore, this deficiency may be counteracted by the addition of lysine and methionine to the autoclaved material (4, 5). Microbiological assays indicate that the heat treatment in question destroys nearly half of the lysine but none of the methionine (6, 7).

Methionine balance studies with chicks reveal that the above heat treatment decreases the availability of methionine (3, 7) and also increases the methionine content of that portion of the protein which is not digested (8). The present investigation was undertaken to determine the nature of the substances which inactivate methionine during autoclaving and the different types of methionine inactivation.

EXPERIMENTAL

Soy bean oil meal,[1] soy bean protein,[2] sucrose,[3] and mixtures of 8 gm. of soy bean protein with 2 gm. of either sucrose, dextrin, agar-agar, gum arabic, glucose, or soy bean oil were the materials studied. 10 gm. of the material, with and without the addition of 0.2 gm. of DL-methionine,[4] were autoclaved for 4 hours at 15 pounds pressure. Untreated soy bean oil meal and soy bean protein served as controls. The same procedures were employed in each of several experiments.

The total methionine content of the above materials was determined, after acid hydrolysis, by microbiological assay with *Leuconostoc mesen-*

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[1] Commercial solvent-extracted soy bean oil meal furnished by Professor J. A. Davidson of the Department of Poultry Husbandry.

[2] The soy bean protein was "alpha" protein furnished by The Glidden Company, Chicago.

[3] Commercial beet sugar.

[4] Synthetic DL-methionine was furnished by The Dow Chemical Company, Midland, Michigan.

teroides by using the medium of Lyman *et al.* (9), which contains oxidized peptone as a source of most of the amino acids. Acid hydrolysates were prepared by autoclaving 1.0 gm. of the material with 20 ml. of 20 per cent hydrochloric acid for 6 hours at 15 pounds pressure. Available methionine in the material was also assayed microbiologically after digesting with the enzymes trypsin and erepsin *in vitro* (10). The percentage of methionine destroyed was calculated from the decreased total methionine

TABLE I

Effect of Autoclaving Soy Bean Protein or Soy Bean Oil Meal on Inactivation of Methionine in Protein

10 gm. portions were autoclaved for 4 hours at 15 pounds pressure. Decreases in the methionine content of the soy bean materials as determined on the acid hydrolysate after autoclaving are considered to be due to destruction of methionine, those determined on the enzymic hydrolysate as due to inactivation, including destruction.

Experiment No.	Soy bean product	Non-protein material added	Treatment	Acid hydrolysis		Enzyme hydrolysis	
				Methionine content	Methionine destroyed	Methionine content	Methionine inactivated
				per cent	*per cent of total*[*]	*per cent*	*per cent of total*[†]
1	Oil meal	None	None	0.54	0	0.48	0
		"	Autoclaved	0.56	0	0.33	32
	Protein	"	None	1.20	0	0.66	0
		"	Autoclaved	1.18	2	0.66	0
		Sucrose[‡]	"	1.11[§]	8	0.02[§]	97
		Dextrin[‡]	"	1.14[§]	5	0.54[§]	18
		Agar-agar[‡]	"	1.05[§]	13	0.62[§]	6
		Gum arabic[‡]	"	1.05[§]	13	0.65[§]	1
2	Oil meal	None	None	0.55	0	0.38	0
		"	Autoclaved	0.55	0	0.40	0
	Protein	"	None	1.17	0	0.67	0
		"	Autoclaved	1.15	2	0.64	4
		Glucose[‡]	"	1.21[§]	0	0.24[§]	64
		Sucrose[‡]	"	1.21[§]	0	0.36[§]	46
		Soy bean oil[‡]	"	1.19[§]	0	0.49[§]	27
		Mixture[‖]	"	1.13[§]	3	0.15[§]	78

[*] Total methionine is considered to be the methionine content of the unautoclaved material determined after acid hydrolysis.

[†] Total methionine is considered to be the methionine content of the unautoclaved material determined after enzyme hydrolysis.

[‡] 8.0 gm. of soy bean protein and 2.0 gm. of non-protein material.

[§] Methionine content in the soy bean protein.

[‖] 5.0 gm. of soy bean protein, 1.0 gm. of sucrose, 1.0 gm. of dextrin, 1.0 gm. of agar-agar, 1.0 gm. of gum arabic, and 1.0 gm. of soy bean oil.

content caused by autoclaving, and the percentage inactivated from the decrease in available methionine.

The mixture of sucrose and methionine, which became syrupy on autoclaving, was dissolved in water and aliquots hydrolyzed as previously described (11). Portions of the materials that contained added oil were extracted with ether in a Goldfisch extractor prior to enzymic digestion *in vitro*, but no differences in available methionine content between the extracted and unextracted samples were observed.

TABLE II

Effect of Autoclaving Methionine in Presence of Soy Bean Oil Meal or Its Constituents

10 gm. portions of the soy bean oil meal, soy bean protein, sucrose, or soy bean oil meal plus non-protein material were used. Methionine was added at a level of 200 mg. of DL-methionine (100 mg. of L-methionine). The samples were autoclaved for 4 hours at 15 pounds pressure.

Experiment No.	Soy bean product	Non-protein material added	Added methionine inactivated		Methionine inactivated but not destroyed
			Acid hydrolysis	Enzyme hydrolysis	
			per cent	*per cent*	*per cent*
1	Oil meal	None	51	51	0
	Protein	"	2	7	5
		Sucrose*	79	74	0
		Dextrin*	32	33	1
		Agar-agar*	42	43	1
		Gum arabic*	19	20	1
2	Oil meal	None	46	43	0
	Protein	"	2	10	8
		Glucose*	69	69	0
		Sucrose*	72	75	3
		Soy bean oil*	24	25	1
		Mixture†	71	71	0
3	None	Sucrose	51	68	17

* 8.0 gm. of soy bean protein and 2.0 gm. of non-protein material.

† 5.0 gm. of soy bean protein, 1.0 gm. of sucrose, 1.0 gm. of dextrin, 1.0 gm. of agar-agar, 1.0 gm. of gum arabic, and 1.0 gm. of soy bean oil.

Results

Soy bean oil meal was changed by autoclaving from a light golden brown to a dark dirty brown color. However, no methionine was destroyed by this treatment, but a variable amount was inactivated (0 to 32 per cent) (Table I). Of the added DL-methionine, approximately 50 per cent was destroyed, with no further inactivation by the heat treatment. All the inactivation of DL-methionine added to any of the materials (except sucrose alone) was due to destruction (Table II).

Soy bean protein was little affected by autoclaving, for the change in color was slight and no inactivation of methionine occurred. However, when the protein was mixed with 20 per cent of sucrose or glucose prior to autoclaving, there resulted a chocolate-brown material with the same total methionine content as before, but with a 46 to 97 per cent loss in availability. About 75 per cent of the added DL-methionine was destroyed.

A less drastic reaction took place when dextrin, agar-agar, or gum arabic was mixed with the protein before autoclaving than when sucrose or glucose was added. A lighter colored material was produced and, except with dextrin (18 per cent), insignificant amounts of methionine were inactivated. Heating with agar-agar or gum arabic destroyed 13 per cent of the protein-bound methionine. Autoclaved mixtures of protein and either gum arabic, dextrin, or agar-agar (19, 32, and 42 per cent) showed smaller losses of added DL-methionine than autoclaved mixtures of protein and sucrose or glucose (75 and 69 per cent).

Autoclaving the mixture of soy bean protein and oil destroyed no protein methionine but inactivated 27 per cent; it destroyed 24 per cent of the added DL-methionine. A mixture of 5 gm. of soy bean protein with 1 gm. each of sucrose, agar-agar, dextrin, gum arabic, and soy bean oil, prepared to simulate the composition of soy bean oil meal, produced no destruction of protein-bound methionine on autoclaving despite the large proportion of carbohydrate, but inactivated 78 per cent; it also caused the destruction of 71 per cent of the added DL-methionine.

An autoclaved mixture of sucrose and DL-methionine gave a viscous syrup, which, when taken up in water, produced a dark reddish brown solution containing some undissolved particles. Furthermore, 51 per cent of the methionine was destroyed and an additional 17 per cent inactivated.

DISCUSSION

Previous observations (6, 7) that autoclaving soy bean oil meal for 4 hours at 15 pounds pressure or for 1 hour at 25 pounds pressure does not destroy methionine have been confirmed. However, the heat treatments in question decreased the amount of microbiologically active methionine released by enzymic digestion *in vitro*. Variable percentages of protein-bound methionine were inactivated by autoclaving soy bean oil meal or a mixture of soy bean protein and sucrose, according to data presented in Table I. Lysine inactivation and destruction were not variable under similar conditions (11).

Sucrose is apparently the component of soy bean oil meal responsible for most of the heat inactivation of protein-bound methionine. Neither gum arabic, an araban, nor agar-agar, a galactan, caused appreciable inactivation, and dextrin caused only 18 per cent. Sucrose, stachyose, arabans, galactans, and dextrin have been reported as the principal carbohydrates of soy bean oil meal (12, 13). Unfortunately, stachyose was not available for use in this experiment. It is improbable that the oil in soy bean oil meal is responsible for any methionine inactivation, because solvent-extracted meal contains but 1.6 per cent (14), and the 20 per cent oil added to the protein was responsible for the inactivation of only 27 per cent of the methionine.

Patton, Hill, and Foreman (15) observed lysine, arginine, and tryptophan to be the only amino acids significantly inactivated by refluxing casein in a 5 per cent glucose solution at 96.5° for 24 hours. However, they measured inactivation by microbiological assay of an acid hydrolysate of the casein, which corresponds to the method of measuring destruction used in the present investigation, and so agrees with the observation that no methionine was destroyed. Whereas we observed that protein-bound methionine was inactivated and free DL-methionine destroyed by autoclaving with either sucrose or glucose, Hill and Patton (16) reported that amino acid inactivation occurred when casein was refluxed with glucose but not with sucrose.

A comparison of the heat inactivation of methionine with that of lysine is of interest. The methionine and lysine in soy bean oil meal were both partially inactivated when the meal was autoclaved, but the reactions differed; methionine was inactivated without any destruction, whereas part of the lysine inactivation was due to destruction. Lysine, either free or protein-bound, was partially inactivated but not destroyed when autoclaved with protein (11), but methionine was not affected. Sucrose caused a destruction of protein-bound lysine but only an inactivation of methionine. Free DL-methionine and DL-lysine hydrochloride were both destroyed when autoclaved with sucrose.

SUMMARY

No destruction of the methionine contained in soy bean protein occurred when soy bean oil meal, soy bean protein, or soy bean protein plus sucrose, glucose, dextrin, or soy bean oil was autoclaved for 4 hours at 15 pounds pressure, but a small amount was destroyed when the protein was autoclaved with agar-agar or gum arabic. None of the DL-methionine mixed with soy bean protein was destroyed, but 24 to 79 per cent of that mixed with soy bean oil meal, sucrose, or the protein plus one of the carbohydrates or soy bean oil was destroyed.

Autoclaving soy bean oil meal caused the binding of a variable amount of its methionine in a form from which biologically active methionine was

liberated by acid hydrolysis but not by enzymic digestion *in vitro*. No inactivation of methionine occurred when soy bean protein was autoclaved by itself, but 46 to 97 per cent less methionine was liberated by enzymic digestion *in vitro* from protein autoclaved with sucrose or glucose than from the unautoclaved protein. Destruction accounted for all of the inactivation of the added DL-methionine.

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METABOLISM OF INORGANIC NITRITE AND NITRATE ESTERS

I. THE COUPLED OXIDATION OF NITRITE BY PEROXIDE-FORMING SYSTEMS AND CATALASE

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This investigation is part of a general study of the metabolism of inorganic nitrite and the nitrate esters of importance in the treatment of cardiovascular disease. Thurlow (1) showed that nitrite could be oxidized by milk peroxidase in the presence of the xanthine oxidase system, and that the reaction was strongly inhibited by catalase. We have found that the rate of oxidation of inorganic nitrite by homogenates of rat liver and kidney was dependent on the content of catalase, and the rate could be increased by additions of crystalline catalase. The further observation that catalase by itself had no effect on nitrite led to the demonstration that the oxidation of nitrite by catalase was coupled to peroxide-forming systems such as xanthine oxidase and D-amino acid oxidase. Catalase was here acting as a peroxidase, in utilizing hydrogen peroxide for the coupled oxidation of nitrite.

Keilin and Hartree (2, 3) were the first to show the peroxidasic activity of catalase in the coupled oxidation of alcohols with either xanthine oxidase, D-amino acid oxidase, or glucose dehydrogenase as the primary oxidation system. The required concentration of catalase was 1000 times that necessary for the ordinary catalytic decomposition of hydrogen peroxide.

Methods

Flavin-adenine dinucleotide was prepared from fresh bakers' yeast according to Warburg and Christian (4). The nucleotide isolated was shown by enzymatic assay (4) to be over 90 per cent pure. D-Amino acid oxidase was purified from hog kidney acetone powder by the isolation procedure and assay method described for sheep kidney by Negelein and Brömel (5). When tested with excess flavin-adenine dinucleotide, the final preparation had an activity of 218 units per mg. of protein, compared with Negelein and Brömel's figure of 187 for material considered by them to be 70 per cent pure.

Xanthine oxidase was purified from cream according to Ball (6), and the preparation contained 70 units per mg. of protein when tested by his procedure. Beef liver catalase was isolated and recrystallized three times as described by Sumner and Dounce (7), and its purity, *Kat. f.*, was 24,000 (8). The catalase content of tissues has been expressed as mg. of catalase of this purity.

The reaction mixtures were incubated in air at 37°, with shaking. Samples were analyzed for nitrite (9) after deproteinization with mercuric chloride and sodium carbonate (10). Nitrate was determined after deproteinization with phosphotungstic acid and removal of chloride (11). The yellow color developed with *m*-xylenol (12) was measured in the Coleman model 6A spectrophotometer at 455 mμ.

TABLE I

Enzymatic Oxidation of Nitrite to Nitrate

500 mg. of liver were homogenized with 4.3 ml. of the following solution: magnesium chloride, 0.01 M; potassium chloride, 0.1 M; phosphate buffer, 0.02 M, pH 7.4. Then 2.83 μM of sodium nitrite were added and the volume was adjusted to 5 ml. The D-amino acid oxidase-catalase system consisted of 18.1 units of D-amino acid oxidase protein, 20 γ of flavin-adenine dinucleotide, 9 mg. of DL-alanine, 3.5 mg. of catalase, and 1.62 ml. of 0.1 M pyrophosphate buffer, pH 8.3, in a total volume of 3 ml. Incubation was at 37°, in air.

Reaction mixture	Incubation time	Initial nitrite	Nitrite disappearance	Nitrate formation
	min.	*μM*	*μM*	*μM*
Liver homogenate	90	2.83	1.88	1.82
" "	90	0.98	0.83	1.01
" "	90	2.83	1.85	1.88
D-Amino acid oxidase-catalase system	75	2.55	2.47	2.38

RESULTS AND DISCUSSION

Oxidation of Nitrite by Tissue Homogenates—Rat liver homogenates oxidized inorganic nitrite, and the quantitative balance between nitrite disappearance and nitrate formation is shown in Table I. The rate of reaction was directly proportional to the concentration of nitrite (Fig. 1). Homogenates of whole blood,[1] kidney, and smooth muscle had 0.25, 0.25, and 0.26, respectively, as much activity as liver. Extracts obtained by centrifuging homogenates of liver for 15 minutes at 13,000*g* in an angle centrifuge were active. They were completely inhibited by 2×10^{-3} M

[1] It is known that nitrites react with hemoglobin to form methemoglobin and nitrosohemoglobin (13). However, the hemoglobin content of liver and kidney homogenates was found to be quite low and was of no significance in determining the rate of disappearance of nitrite in the presence of these tissues.

cyanide, 4 × 10^{-3} M mercuric chloride, heating at 80° for 10 minutes, and in the absence of oxygen.

Coupled Oxidation of Nitrite by D-*Amino Acid Oxidase and Catalase*—Dialysis of rat liver extracts resulted in reduced nitrite-oxidizing activity, which could be restored by additions of xanthine or DL-alanine. It was then found that oxidation of nitrite occurred in a simple reconstructed system composed of D-amino acid oxidase protein, flavin-adenine dinucleotide, DL-alanine, and catalase. As seen in Fig. 2, the rate of nitrite oxidation is proportional to the concentration of D-amino acid oxidase up to a point when catalase becomes limiting. With catalase as the rate-limiting enzyme a straight line results from a plot of the first order velocity constant for nitrite oxidation, $k_{\mathrm{NO}_2^-}$, against the concentration of catalase (Fig. 3).[2]

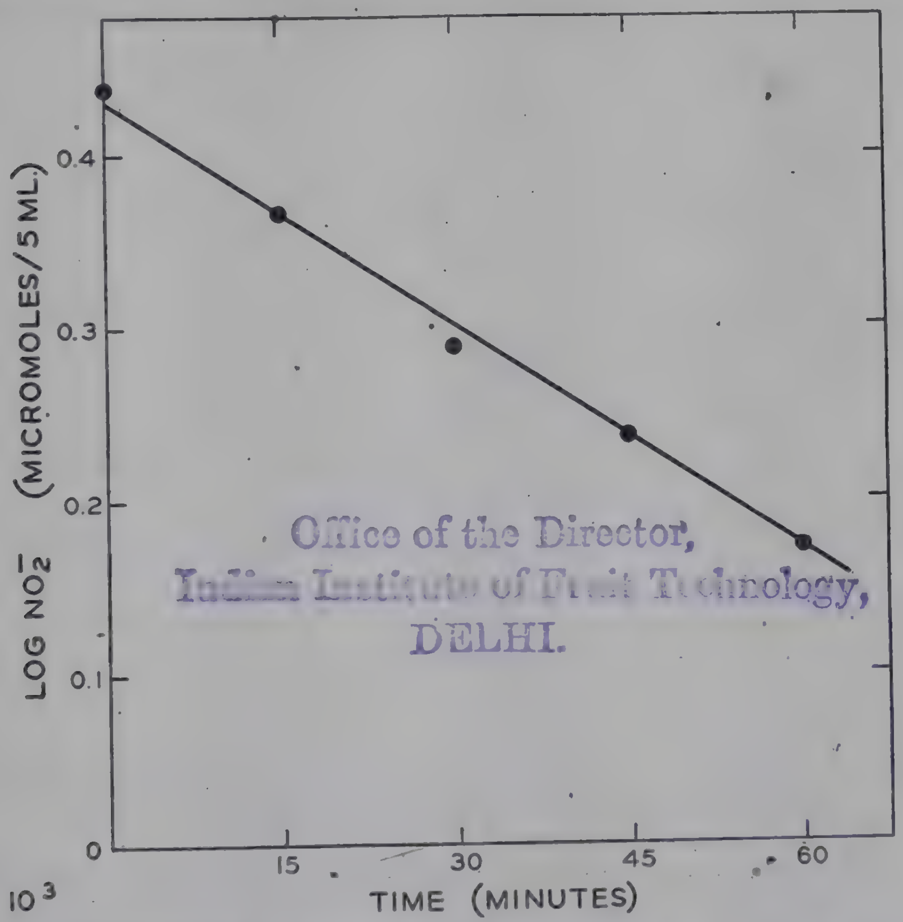

FIG. 1. Disappearance of nitrite when incubated with rat liver homogenate. Test conditions as described in Table I.

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The coupled oxidation could also be shown manometrically with the purified D-amino acid oxidase system described in Table I. Incubations

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[2] The higher rate of nitrite oxidation shown in Fig. 3, as compared with Fig. 2, is due to the establishment of a more favorable pH for the coupled oxidation.

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were carried out in Warburg flasks with 0.2 ml. of 10 per cent potassiu hydroxide in the center well. The final oxygen uptake was 345 μl. wi the purified D-amino acid oxidase system, with 3 mg. of DL-alanine. Th was reduced to 187 μl. by the addition of 3 mg. of catalase. In the pre ence of 5 mg. of sodium nitrite plus 3 mg. of catalase, the total oxyg

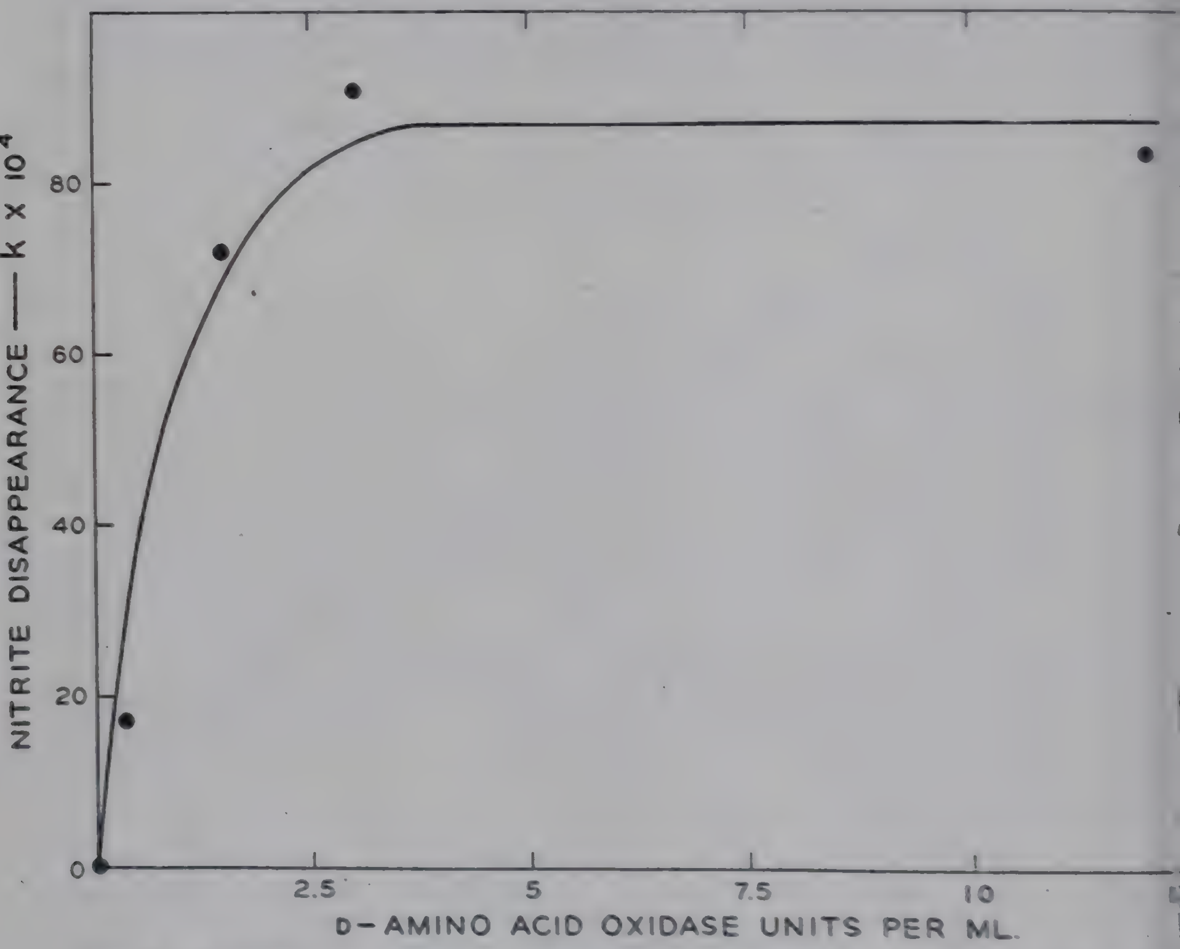

FIG. 2. Effect of concentration of D-amino acid oxidase protein on rate of nit oxidation, with catalase fixed at 1.2 mg. per ml. Test conditions as describe Table I. The first order velocity constant, k, equals $(-d \log NO_2^-)/(dt)$. Eno flavin-adenine dinucleotide has been added to saturate the D-amino acid oxid at all concentrations.

consumption was 300 μl. Catalase and D-amino acid oxidase had effect on sodium nitrite in the absence of DL-alanine.

Coupled Oxidation of Nitrite by Xanthine Oxidase and Catalase—C dation of nitrite by the xanthine oxidase system plus catalase was de onstrated with the purified oxidase enzyme as well as with crude pre rations (Table II). The data shown are representative of ten separ experiments. No reaction occurred when hypoxanthine was omitt With relatively large amounts of catalase there was a rapid disappeara nitrite. Without catalase there was some oxidation of nitrite because the presence of milk peroxidase in the xanthine oxidase preparation. s expected, small concentrations of catalase inhibited the oxidation by talyzing the decomposition of hydrogen peroxide into oxygen and water, hus interfering with the action of milk peroxidase (1, 14).

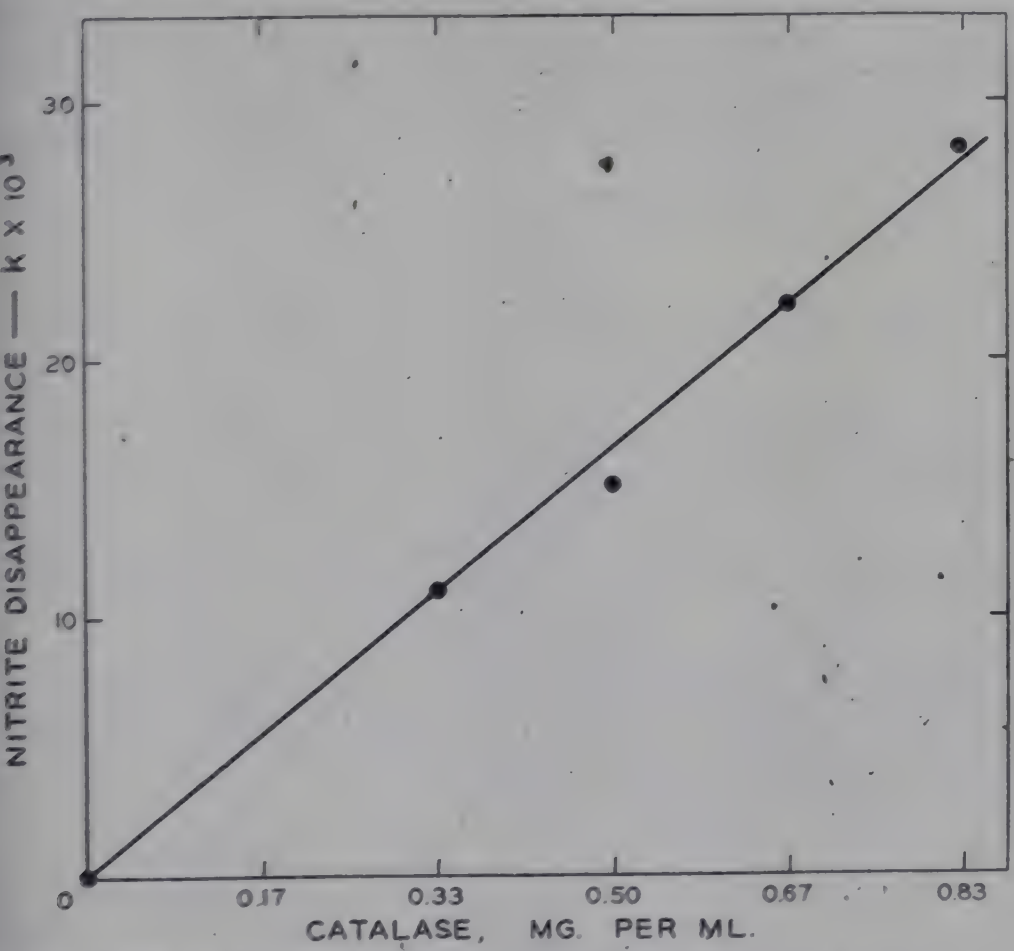

Fig. 3. Effect of catalase concentration on rate of nitrite oxidation. Total olume, 5 ml.; temperature, 37°; gas phase, air; samples taken for analysis at 30 nd 75 minutes. Additions to each flask: 2.5 ml. of 0.067 M phosphate buffer, pH 33; 20 γ of flavin-adenine dinucleotide; 13.5 mg. of DL-alanine; 27.1 units of D-mino acid oxidase protein; 0.2 mg. of sodium nitrite; catalase. The rate of nitrite xidation is obtained by calculating the first order velocity constant, $k = (-d$ og $NO_2^-)/(dt)$.

Dependence of Rate of Nitrite Oxidation by Tissue Homogenates on Their ontent of Catalase—Since rat liver and kidney contain both peroxide-orming oxidase enzymes and catalase (15–17), it was a question which f these would limit the rate of nitrite oxidation. Table III shows that atalase was limiting. The liver homogenate contributed 0.16 mg. of atalase per ml. of reaction mixture and the velocity constant for nitrite

TABLE II

Coupled Oxidation of Nitrite by Xanthine Oxidase and Catalase

The flasks contained 5.8×10^{-4} M sodium nitrite, 0.037 M phosphate buffer, p 7.39, and 1.5×10^{-3} M hypoxanthine. Incubation was at 37°, in air. Samples wer removed for analysis at 30 and 75 minutes.

Experiment No.	Units xanthine oxidase per ml. reaction mixture	Concentration of catalase per ml. reaction mixture	Rate of nitrite oxidation, $\frac{-d \log (NO_2^-)}{dt}$
		mg.	*min.*$^{-1}$ × 10^3
1	20	0	8.0
		0.006	1.2
		0.025	1.0
		1.7	24
		3.2	36
2	2.1	0	0.5
		1.9	2.1
		3.9	6.6

TABLE III

Effect of Catalase and of D-*Amino Acid Oxidase System on Oxidation of Nitrite b Rat Liver Homogenate*

Each flask contained homogenate from 500 mg. of liver with a catalase content o 0.79 mg. Flasks with D-amino acid oxidase protein contained in addition 30 γ o flavin-adenine dinucleotide and 13.5 mg. of DL-alanine. Total fluid volume, 5 ml. phosphate buffer, 0.02 M, pH 7.33; magnesium chloride, 0.01 M; potassium chloride 0.1 M; temperature, 37°; gas phase, air.

Reaction mixture	Nitrite			Rate of nitrite oxidation, $k_{NO_2^-}$	Rate ($k_{NO_2^-}$) per unit catalas concentration
	0 min.	30 min.	75 min.		
	μM	*μM*	*μM*	*min.*$^{-1}$ × 10^3	$\frac{ml.}{mg. \times min.} \times 1$
Homogenate	2.83	2.02	1.32	4.0	2.6
" + D-amino acid oxidase protein					
9.0 units	2.83	1.94	1.10	5.4	3.4
27.2 "	2.83	1.98	1.10	5.4	3.4
Homogenate + 3.5 mg. catalase	2.83	1.00	0.20	15.0	1.7
Catalase alone (3.5 mg.)	2.83	2.83	2.83	0.0	0.0

oxidation, $k_{NO_2^-}$, was 4.0×10^{-3} min.$^{-1}$. The rate of nitrite oxidatio per unit concentration of catalase was then

$$\frac{4.0 \times 10^{-3} \text{ min.}^{-1}}{0.16 \text{ mg. per ml.}} \quad \text{or} \quad 2.5 \times 10^{-2} \frac{\text{ml.}}{\text{mg.} \times \text{min.}}$$

With purified D-amino acid oxidase in a reconstructed system, the figure was 3.4×10^{-2} (ml.)/(units × minutes). Addition of the components of the D-amino acid oxidase system had little influence on the rate of nitrite oxidation by liver homogenates. On the other hand, additions of crystalline catalase stimulated nitrite oxidation greatly.

A similar dependence on catalase concentration was observed in comparable experiments with kidney homogenates (Table IV). Their catalase content was one-fourth that of liver and this accounts for the fact that the rate of nitrite oxidation by kidney homogenates was only one-fourth that of liver.

TABLE IV

Effect of Catalase and of D-Amino Acid Oxidase System on Oxidation of Nitrite by Rat Kidney Homogenate

Each flask contained homogenate from 500 mg. of kidney with a catalase content of 0.25 mg. The addition of the D-amino acid oxidase system included 18.1 units of D-amino acid oxidase protein, 20 γ of flavin-adenine dinucleotide, and 13.5 mg. of DL-alanine. Total fluid volume, 5 ml.; phosphate buffer, 0.02 M, pH 7.33; magnesium chloride, 0.01 M; potassium chloride, 0.1 M; temperature, 37°; gas phase, air.

Reaction mixture	Nitrite			Rate of nitrite oxidation, $k_{NO_2^-}$	Rate ($k_{NO_2^-}$) per unit catalase concentration
	0 min.	30 min.	75 min.		
	μM	*μM*	*μM*	*min.*$^{-1}$ × 10^3	$\frac{ml.}{units \times min.} \times 10^2$
Homogenate	2.75	2.57	2.26	1.0	1.9
" + D-amino acid oxidase system	2.75	2.38	2.05	1.4	2.9
Homogenate plus catalase					
1.7 mg.	2.75	2.09	1.23	4.4	1.2
2.5 "	2.75	1.93	0.88	6.2	1.2

Reduction of Nitrite by Liver Homogenate—Bernheim and Dixon (18) have demonstrated the enzymatic anaerobic reduction of nitrate to nitrite in liver. This has been confirmed, but it was found that the anaerobic reduction of nitrate was slight as compared with the aerobic oxidation of nitrite. After incubating a 10 per cent liver homogenate containing 0.1 M sodium nitrate for 105 minutes, the final concentration of nitrite was only 18×10^{-5} M. In a similar experiment with 0.01 M sodium nitrate the accumulation of nitrite was 2×10^{-5} M, which represents only 0.2 per cent reduction. Table I shows that on aerobic incubation of liver homogenate with 6×10^{-4} M sodium nitrite 64 per cent was oxidized to nitrate in 90 minutes.

SUMMARY

1. The oxidation of nitrite to nitrate was catalyzed by the D-amin acid oxidase or xanthine oxidase system acting in conjunction with cat lase. In this reaction hydrogen peroxide formed by the oxidase was ut lized by catalase for the coupled oxidation of nitrite.

2. The mechanism of nitrite removal by rat liver and kidney hom genates was shown to be dependent on catalysis of coupled oxidation catalase. The rate of reaction could be correlated with the catalase co tent of the different tissue preparations. Also, additions of crystalli catalase to homogenates increased the rate of nitrite oxidation, ev though catalase by itself had no effect.

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THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

I. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF ORGANIC ACID SALTS

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It was observed in this laboratory about 12 years ago (1) that, when xcised tobacco leaves are placed with their bases in water and kept in arkness for 2 or 3 days, the malic acid content decreases and that of citric cid increases, the total organic acidity of the tissues remaining substan- ally unchanged (2). Under these circumstances, malic acid apparently ndergoes transformations that result in the formation of citric acid. he reaction is quantitatively an important one, for 12 per cent or more of he organic solids of the leaf may be involved. Similar observations have lso been recorded by Pyatnitskii (3).

No explanation of the chemical mechanism of the reaction could be ffered at that time, save the speculation that pyruvic acid could be upposed to condense with oxalacetic acid (both being assumed to be derived from malic acid) with the eventual formation of citric acid. This eaction had been previously investigated by Knoop and Martius (4). oday, many different enzymatic transformations of organic acids have een described and such reactions are known to occur in a wide variety of nimal as well as plant tissues. Certain of the reactions have been organ- ed into systems of which the Krebs tricarboxylic acid cycle, advanced riginally to account for the respiration of muscle, is the outstanding xample (for reviews see (5, 6)). That a system of enzymatic reactions milar to or, possibly, identical with the Krebs cycle does in fact operate plant tissues has been rendered highly probable by such findings as those Machlis (7) on the respiration of barley roots, of Bonner and Wildman) on the respiration of spinach leaves, and of Bonner (9) on the respiration *Avena* coleoptile. However, in the plant leaf, the reactions, enzymati- lly controlled or not, in which organic acids may become involved are rtainly not restricted to those defined by the Krebs cycle. The relatively enormous quantity of acids present (from about 5 to, in ex cases, 40 per cent or more of the organic solids, 15 to 25 per cent commonly found) may be cited in support of this view. Accordin has seemed desirable to carry out tests of the capacity of a leaf to about transformations of organic acids with the object of obtaining tative information regarding the enzymatic reactions that may occu

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* Deceased, November 20, 1947.

† Deceased, November 20, 1948.

To this end, leaves cut from specially grown tobacco plants were with their petioles in 0.2 M solutions of the potassium salts of a num organic acids and kept in total darkness for 2 days. The leaves wer dried and the organic acidity, together with the malic, citric, and acid content, was determined. Leaves cultured on water or on solutions of magnesium chloride were used as controls to assist in judg of the relative magnitude of the changes that occurred. The method to introduce organic acids into leaf tissues is to be prefe vacuum infiltration for the present purpose because the gas spaces leaves are undisturbed and the substance under study is added throughout the experimental period rather than all at once.

EXPERIMENTAL

Organic Acids of Tobacco Leaves—The composition of the Ro strain of *Nicotiana tabacum* grown on nitrate, employed for one g the present experiments, is typical of that of freshly picked tobacc grown under normal agricultural conditions. These contained 1 cent of the organic solids as malic acid, 2.2 per cent as citric a per cent as oxalic acid, and 3.5 per cent as unknown acids, the las arbitrarily calculated as citric acid. The total was 23.3 per cen unknown acids doubtless comprise a complex group; small propor succinic acid have been detected in tobacco leaves with certainty (1 the literature contains claims for the presence of fumaric acid (11) as of caffeic, quinic, and chlorogenic acids (for the literature se Whether pyruvic, oxalacetic, α-ketoglutaric, aconitic, or other bio related acids are present in detectable quantities has not as y established.[1]

If, as a first assumption, it is supposed that the organic acids mally present in an equilibrium relationship together with the enzy coenzymes necessary to provide for the various transformations occur, the conditions in the tobacco leaf are obviously such that m is present in a high concentration with citric, oxalic, succinic, a acids in relatively low concentrations (13, 14). When abnorma tions are imposed as, for example, when the leaf is detached and p continuous darkness, metabolic reactions continue, but the path lowed may be presumed to depart from the normal. However, the rved changes in composition that supervene illustrate some, at least, e reactions that can take place. If, in addition, organic acid anions ntroduced, the metabolic pathway will again differ, provided that the mes present are capable of acting upon the added substances. But e the added substance is, as it were, foreign to the enzyme systems of ells and is non-toxic, it would be expected to accumulate without ing any substantial effect upon the regular course of events in its nce.

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[1] Tests recently carried out in this laboratory indicate that there is no si quantity of isocitric acid in tobacco leaves.

eparation of Tobacco Plants—It appears to be a general rule that, plants are grown with most or all of the nitrogen supplied in the form mmonium ions, the organic acid content of the leaves is depressed in parison with the leaves of plants grown with all of the nitrogen supplied trate. This observation has been made with tomato (15), tobacco *Bryophyllum* (17), and, with respect to malic acid, with narcissus (18). tobacco plant shows the effect remarkably well and, in planning the nt experiments, it therefore seemed desirable to test the effect of re of the excised leaves in solutions of organic acids not only upon s of normal acid composition, but also upon leaves the acids of which been diminished in initial concentration by growth of the plants upon onium salts.

e varieties chosen were the Rosenberg strain of *Nicotiana tabacum* and Brazilia variety of *Nicotiana rustica* because the leaves of these varie- are provided with short petioles and are more convenient for excised culture work than the sessile leaves of the more commonly grown s. The plants were grown in sand in the greenhouse, one lot of each ty receiving a culture solution that contained all of its nitrogen as te, the second lot receiving one that contained 75 per cent of the gen as ammonium ion and 25 per cent as nitrate (for details of the position of the solutions see (17)). The composition of the second re solution was chosen as the result of earlier experiments which ed that plants of normal size can be obtained with it, although stunt- f growth may occur if higher proportions of ammonium nitrogen are oyed (16). Growth was normal in all cases and there was little differ- in the size or appearance of the plants on the two culture solutions.

mpling and Culture—The samples of leaves were taken from the plants e Rosenberg strain 57 days after transplantation at the time the rs were beginning to develop, twenty leaves being cut from each of n plants grown on nitrate as the source of nitrogen and from each of lants grown with 75 per cent of the nitrogen supply as ammonium ion. revity, these will be referred to, respectively, as nitrate and ammonia s (or leaves).

e leaves from the *rustica* plants were collected 63 days after transplantation, ten leaves being cut from each of eleven nitrate plants and te ammonia plants. The leaves in each of the four lots were repeatedl shuffled to provide for randomization with respect to plant of origin an position on the plant, and seventeen-leaf samples from the Rosenber plants and ten-leaf samples from the *rustica* plants were selected. Th initial fresh weights of the samples are shown in Table I. The fundamental assumption is that each sample has the same initial compositi per unit of fresh weight. Control samples of each kind were at once dri for analysis and the rest were arranged in V-shaped troughs, the sides which supported the leaves, while the petioles were held in position at t bottom of the trough by a glass rod. Each trough contained a sample nitrate leaves arranged along one side and one of ammonia leaves along t

Table I

Fundamental Data on Samples of Excised Tobacco Leaves Subjected to Culture in 0.2 M Solutions of Salts

The data are expressed in gm. per sample.

Culture solution	*N. tabacum*, Rosenberg strain, 17 leaves						*N. rustica* var. Brazilia, 10 leaves					
	Nitrate plants			Ammonia plants			Nitrate plants			Ammonia plants		
	Fresh weight		Crude dry weight	Fresh weight		Crude dry weight	Fresh weight		Crude dry weight	Fresh weight		Crude dry
	Before culture	After culture		Before culture	After culture		Before culture	After culture		Before culture	After culture	
Control	263.6		24.2	236.4		20.0	231.2		18.8	193.0		16
"	260.5		22.5				232.6		17.7			
Water	263.5	278.5	23.5	238.9	254.1	17.5						
Magnesium chloride	264.4	269.2	25.9	234.5	238.4	22.0	231.7	241.9	20.1	190.7		17
Magnesium nitrate	261.5	255.9	24.7	235.9	234.9	23.1						
Ammonium sulfate	264.8	253.2	21.5	234.0	231.9	21.9						
Malate	264.6	279.8	27.1	234.5	248.3	25.0						
Citrate							232.3	231.2	22.0	188.0	195.3	19
Ammonium citrate							230.0	220.5	19.3	189.2	186.6	17
Succinate	261.7	279.5	26.9	239.6	257.1	24.9	231.7	258.7	22.7	193.4	217.5	19
Fumarate							230.7	246.5	21.3	190.5	213.2	19
Pyruvate	265.0	220.6	25.4	236.0	160.0	21.5	232.2	230.4	20.5	190.7	144.0	17
Fumarate + malonate	260.7	237.0	25.7	234.6	163.6	20.2	233.0	252.1	23.8	191.7	116.2	17
Lactate							232.8	186.8	22.2	189.2	139.7	19
Tartrate	260.0	266.2	25.7	237.7	244.8	22.8						
Malonate							230.7	173.7	22.0	192.1	116.2	18

other. The troughs were all in position in a completely dark room (temperature 25 ± 1°) within about 2 hours from the time of cutting the leaves. The culture solutions were added to the troughs in sufficient amount to cover the ends of the petioles, about 1300 ml. being required per trough.

The culture solutions were all 0.2 M in concentration, this having been found by preliminary trials with ammonium sulfate solution to be the maximum that the leaves could tolerate without developing necrotic lesions and becoming entirely flaccid within 48 hours. The solutions were prepared from weighed quantities of the free acids and were neutralized to approximately pH 5.5 with 5 N potassium hydroxide, or with ammonium hydroxide in the case of one of the citric acid solutions. The solution of mixed malonate and fumarate was 0.1 M with respect to each acid. In addition to the tests with organic acid culture solutions, tests with magnesium chloride were carried out on all four lots of leaves, in part to serve as a control and in part to study certain effects reported by Pyatnitskiĭ, and with magnesium nitrate, ammonium sulfate, and water on the Rosenberg leaves, in order to obtain information on the nitrogen metabolism.

At the end of the period of culture, the leaves were rinsed off, weighed, and dried in a current of air in an oven at 80°. Because of the time necessary to handle the samples one by one, the length of time of culture varied from 45 to 46 hours for the Rosenberg leaves and from 47 to 49 hours for the *rustica* leaves. The crude dry weights recorded in Table I represent the weight after each sample had been dried but before it had been broken up and ground for analysis. The analytical determinations were made upon the dry powder and the results were then calculated, with the aid of factors derived from the data in Table I, to give the quantity that would have been obtained if each sample had weighed exactly 1 kilo at the start of the experiment.

A general idea of the condition of the leaves after being subjected to culture may be obtained from the data in Table I for fresh weight before and after treatment. Where there was a substantial increase in fresh weight, absorption of solution was clearly greater than the transpiration loss and the leaves showed this by their unusual turgidity. Where the weights differed by only a few gm., there was no striking effect upon the turgidity. Where there was a marked loss in weight, as with lactate and malonate, the leaves were completely flaccid. In some cases (ammonium sulfate, citrate, pyruvate, lactate, and malonate), there was more or less evidence of chlorophyll degeneration, the color having become paler green, and occasional leaves were mottled. On the whole, however, the leaves on most of the culture solutions survived the treatment without notable evidence of harm; they were still living and metabolizing organisms.

Effect of Culture on Solids and Ash

Tables II and III show the details of the changes in composition of t samples brought about by the treatment, the data referring to samples o kilo initial weight. The first column of data in both Tables II and I shows the composition at the start.

Culture in water produced remarkably turgid leaves (19) but there w no significant change in the ash. The loss of 3.5 gm. of organic solids fr the Rosenberg nitrate leaves represents the effect of respiration; it amou to 5.0 per cent of the organic solids and agrees closely with a previously ported value (19) obtained after 49 hours of culture. However, the Ros berg ammonia leaves showed an apparent loss in inorganic solids and grea loss in organic solids after culture in water than was noted with the nitr leaves. This observation illustrates one of the difficulties with exp ments on excised leaves. In spite of the greatest pains taken to prov for randomization in the selection of the samples, erratic differences casionally show up when the leaves are analyzed. The sampling erro sometimes large and, in order to obtain results of high significance, ma fold replication of the individual samples and statistical analysis of results would be required. This was not done in the present prelimin study because of the greatly increased amount of analytical work involv General experience has shown that changes of the order of 10 to 15 per c or more are likely to be valid while small changes may be open to the s picion that they represent, in part at least, errors in sampling.

Although the increases in fresh weight were in no case large and th were a number of cases in which the over-all loss in fresh weight was s stantial, the increase in inorganic solids demonstrates that apprecia quantities of culture solution entered the leaves. The only exceptions the tests with ammonium sulfate and ammonium citrate, neither of wh could contribute to the ash content. The magnesium salts did not fact, enter in large amounts; the ash weights of the samples cultured magnesium chloride increased only about 30 per cent on the aver The potassium salts of the organic acids, however, were freely taken for the ash weights increased markedly. Pyruvate was apparently least readily absorbed, the increase in ash weight being in three cases than 50 per cent; citrate, succinate, and fumarate, on the other hand, w absorbed to such an extent that the ash weights increased more than per cent. The average (twenty-four cases) was 76 ± 9 per cent.

Although small losses of organic solids by respiration are to be ant pated in experiments of this type, accumulation of organic solids observed in all save two cases among the nitrate plants. The Rosenb ammonia plants also showed gains in three of five cases. However, The data are expressed in gm. per kilo of initial fresh weight.

N. tabacum var. Rosenberg	Weight before culture	Changes in composition after 46 hrs.								
		Water	Magnesium chloride	Magnesium nitrate	Ammonium sulfate	DL Malate	Succinate	L(*dextro*)-Tartrate	Pyruvate	Fumarate + malonate
Nitrate plants										
Fresh weight	1000	+57.0	+18.0	−21.0	−44.0	+58.0	+69.0	+23.0	−167	−90.0
Inorganic solids	15.2	+0.31	+3.97	+1.50	+0.43	+11.2	+7.93	+8.77	+7.34	+11.1
Organic solids	69.5	−3.50	+2.00	+1.92	−8.00	+3.27	+0.16	+1.30	−0.40	+0.35
Ammonia plants										
Fresh weight	1000	+64.0	+16.0	−4.0	−9.0	+59.0	+75.0	+30.0	−322	−303
Inorganic solids	16.7	−1.5	+4.7	+2.3	0.0	+13.6	+12.0	+9.6	+8.2	+8.6
Organic solids	63.6	−9.8	+0.1	+3.2	+6.7	+6.5	+3.2	+0.9	−3.7	−9.2

TABLE III

Effect of Culture on Various Salts upon Solids and Ash of Excised Tobacco Leaves

The data are expressed in gm. per kilo of initial fresh weight.

N. rustica	Weight before culture	Changes in composition after 48 hrs.								
		Magnesium chloride	Citrate	Ammonium citrate	Lactate	Malonate	Succinate	Fumarate	Pyruvate	Fumarate + malonate
Nitrate plants										
Fresh weight	1000	+44.0	−5.0	−41.0	−198	−248	+116	+66.0	−8.0	+81.0
Inorganic solids	13.0	+5.1	+13.0	0.0	+12.6	+11.6	+13.9	+13.9	+8.0	+14.4
Organic solids	60.9	−0.9	+0.1	+4.6	+2.1	+3.0	+6.1	−0.8	+0.5	+6.8
Ammonia plants										
Fresh weight	1000		+39.0	+9.0	−244	−395	+125	+119	−245	−394
Inorganic solids	15.4	+4.4	+14.1	−0.3	+13.9	+8.6	+14.1	+13.5	+7.0	+10.7
Organic solids	67.1	−5.2	−0.9	+3.6	−0.8	−0.7	−2.3	+0.7	−6.7	−10.6

TABLE IV

Effect of Culture on Various Salts upon Organic Acids of Excised Tobacco Leaves

The data are expressed in milliequivalents per kilo of initial fresh weight.

N. tabacum var. Rosenberg	Weight before culture	Changes in composition after 46 hrs.								
		Water	Magnesium chloride	Magnesium nitrate	Ammonium sulfate	DL-Malate	Succinate	L(*dextro*)-Tartrate	Pyruvate	Fumarate + malonate
Nitrate plants										
Total organic acids	242	+2.5	−6.0	−26.0	−60.0	+142	+126	+86.0	+59.0	+125
Oxalic acid	21.5	−2.0	+0.76	+0.45	−1.33	+6.3	+4.8	−0.5	+2.6	−1.9
Citric "	23.7	+12.6	+29.8	+17.5	+1.1	+37.6	+46.7	+25.8	+49.6	+43.9
Malic "	168	−18.0	−44.0	−56.0	−73.0	+68.0	+40.0	−57.0	−41.0	−88.0
Unknown acid	29.2	+9.7	+7.7	+12.0	+13.1	+30.3	+34.4	+118	+47.7	+171
Soluble carbohydrate, *gm*.	3.2	−1.1	−0.9	−1.3	−2.3	−0.8	−0.7	−1.4	−1.0	−2.0
Ammonia plants										
Total organic acids	74.0	−11.3	−0.2	−7.3	+0.5	+182	+163	+122	+53.6	+121
Oxalic acid	11.8	−1.4	+0.6	−0.5	−1.5	+3.4	+3.0	+0.4	+1.3	−0.2
Citric "	3.4	−0.4	+0.3	+0.5	−0.3	+40.4	+48.2	+4.7	+12.6	+10.7
Malic "	12.1	−5.6	−5.4	−3.4	−1.9	+142	+102	+0.7	+0.8	+6.4
Unknown acid	46.7	−4.0	+4.2	−3.9	+4.1	−3.5	+9.5	+116	+38.8	+105
Soluble carbohydrate, *gm*.	3.4	−2.1	−1.7	−1.9	−2.4	−1.0	−1.3	−2.0	−2.6	−2.7

ustica ammonia plants lost organic solids in six out of seven cases, the ulture on ammonium citrate being the exception. Interpretation of these lifferences in behavior is scarcely possible in the absence of data on the elative rate of gas exchange, but it may be pointed out that by far the reater part of the changes in fresh weight consisted in the gain or loss of vater and that the increases in organic solids represent absorption of the rganic acid anions; this usually more than compensated for the loss by espiration.

Effect of Culture on Inorganic Salts upon Organic Acid Content

Tables IV and V show the effect of the treatment upon the organic acid omposition of the leaves. The first column of data shows the composition f the control samples at the start. The subsequent columns show the hanges in composition brought about by the treatment, the units being nilliequivalents of acid computed for a sample that weighed 1 kilo at the start. The first five columns of figures in Table IV show the effect of ulture of Rosenberg leaves on water and on 0.2 M solutions respectively f magnesium chloride, magnesium nitrate, and ammonium sulfate. The irst two columns of Table V show the effect upon *rustica* leaves of culture pon magnesium chloride.

The normal course of the metabolism of excised tobacco leaves, that is to say normal in the sense that no reagent that affects the course of events s introduced, is illustrated by the results of the culture of the Rosenberg itrate leaves in water. Citrate acid increased by about 50 per cent and malic acid decreased by about 11 per cent. Nevertheless, the total organic acids did not change significantly and the increase in unknown organic acids (calculated by difference) was so small as to be negligible. This general type of behavior was duplicated in the leaves cultured on magnesium chloride, although in this case the increase in citric acid and the loss f malic acid were appreciably greater than in the sample cultured on water.

Comparison of the acid composition of the Rosenberg ammonia leaves vith that of the nitrate leaves clearly shows the effect of the conditions nder which the plants were grown. The total acidity and the oxalic, itric, and malic acid contents were greatly depressed, although the quantity of unknown acids was considerably higher than in the nitrate leaves. This is also true for the *rustica* leaves (Table V). Culture of the Rosenberg ammonia leaves on water brought about no definite change in citric cid but there was a large relative decrease in malic acid. The significance f the apparent drop in total acid is not clear inasmuch as this sample was ow in inorganic solids (see Table II) and therefore may not have been nitially of exactly the same composition as the control. However, the

Table V

Effect of Culture on Various Salts upon Organic Acids of Excised Tobacco Leaves

The data are expressed in milliequivalents per kilo of initial fresh weight.

N. rustica	Weight before culture	Changes in composition after 48 hrs.								
		Magnesium chloride	Citrate	Ammonium citrate	Lactate	Malonate	Succinate	Fumarate	Pyruvate	Fumarate + malonate
Nitrate plants										
Total organic acids	177	−10.0	+171	+83.0	+132	+162	+180	+146	+66.0	+218
Oxalic acid	6.8	+0.3	+1.4	+3.2	+1.0	+0.1	+2.7	+1.7	+1.9	−1.4
Citric "	17.4	+16.9	+84.8	+48.0	+44.0	+6.4	+50.7	+56.5	+42.1	+33.2
Malic "	110	−32.0	+90.4	+40.0	+14.9	−66.9	+68.0	+62.4	−4.8	−28.1
Unknown acid	42.3	+4.5	−6.0	−7.9	+72.3	+222	+58.5	+25.7	+26.7	+215
Soluble carbohydrate, *gm.*	2.6	−1.7	−1.5	−2.0	−1.5	−1.2	−1.1	−1.8	−1.3	−1.2
Ammonia plants										
Total organic acids	101	−14.0	+181	+103	+158	+104	+195	+162	+40.0	+134
Oxalic acid	0.7	+3.9	+2.8	0.0	+0.1	+0.1	+0.2	+0.2	−0.2	−0.1
Citric "	3.2	+2.6	+65.4	+51.5	+40.1	0.0	+50.7	+40.4	+18.6	+12.0
Malic "	31.9	−12.2	+67.2	+39.5	+7.9	−12.1	+88.5	+56.1	−11.1	−6.4
Unknown acid	65.2	−7.9	+45.1	+11.8	+110	+117	+55.6	+65.7	+32.8	+128
Soluble carbohydrate, *gm.*	3.1	−2.34	−2.60	−2.86	−2.25	−2.36	−1.9	−2.3	−2.6	−2.4

behavior of the sample cultured on magnesium chloride was closely similar and in this case there was no change in total acidity.

The test with magnesium chloride on the Rosenberg nitrate leaves is especially interesting in view of the observations of Pyatnitskiĭ (20). In experiments designed to promote the formation of citric acid in tobacco leaves to a maximal extent, this investigator noted that culture on magnesium chloride was more effective than culture on any other of a series of inorganic salts. The citric acid content was rather more than doubled and, if the leaves were subsequently cured for 2 days, was increased as much as 4-fold. The present test with Rosenberg nitrate leaves apparently confirms Pyatnitskiĭ's observation that magnesium chloride has a special effect upon the synthesis of citric acid, but it should be noted that the Rosenberg ammonia leaves did not behave in a similar manner. Further tests of this point are obviously desirable.

The data for the *rustica* leaves in Table V show that the leaves of this species, when cultured on magnesium chloride solution, behave in general like those of the Rosenberg strain. There was again an increase in citric acid and a decrease in malic acid, coupled with only a small if not negligible change in total acidity. The effect of growth of *rustica* plants on ammonium salt culture solutions as compared with growth on nitrate was, on the whole, less profound than in the Rosenberg plants. However, in both species, the ammonia plants are characterized by having about two-thirds of the total acidity present as unknown acids. Culture of these leaves on magnesium chloride gave rise to proportionately large changes in citric and malic acids in the same direction as those observed in the nitrate leaves and, in addition, brought about a sharp increase in oxalic acid.

Culture of the Rosenberg nitrate leaves on ammonium sulfate gave results somewhat different from those discussed hitherto. There was a definite loss of total acidity, a small and probably not significant gain in citric acid, and a major loss of malic acid. Accordingly no evidence for the conversion of malic to citric acid was found in this case; rather, the change seems more like that observed when the plants are grown in ammonium salt culture solutions; that is, the general effect is in the direction of lower total acid content. This may have obliterated any analytical evidence for the conversion of malic to citric acid. The Rosenberg ammonia leaves showed no significant effect of culture on ammonium sulfate.

Effect of Culture on Organic Acids upon Organic Acid Content

Two entirely independent groups of analytical data demonstrate that organic acid anions entered the leaf tissues during culture; these are the increases in ash and the increases in total organic acidity. The changes in total acidity shown in Tables IV and V range from 40 m.eq. for the *rustica* ammonia leaves cultured on pyruvate to 218 m.eq. for the *rustica* nitrate leaves cultured on the mixture of fumarate and malonate. The average of the twenty-four cases was 135 ± 47 m.eq.; calculated arbitrarily as malic acid, this is the equivalent of 9 gm. of acid taken up per sample or about 13 per cent of the organic solids of the leaves at the start. Despite the variation among the different acids, the effect was therefore, on the whole, considerable.

The increase in total acidity is closely correlated with the increase in ash. This increase may be assumed to represent essentially potassium carbonate, and the coefficient of correlation with the increase in organic acidity in the twenty-four cases was 0.90. The probability is less than one in a billion that this is a chance relationship.[2]

The special value, for experiments of this type, of the leaves of the ammonia plants can be appreciated from the data for the increases in total acidity. The Rosenberg ammonia leaves showed increases of 173 per cent over the controls and the *rustica* ammonia leaves 133 per cent as the average of the experiments with the two types. The nitrate leaves on the other hand increased by only 44 and 82 per cent in organic acidity respectively. The ammonia leaves, with their lower initial total acidity, were thus the more strikingly affected by the imposed conditions.

DL-*Malate*—When cultured on potassium malate, the total organic acidity in the Rosenberg nitrate leaves increased by 142 m.eq., citric acid by 37.6, and unknown acids by 30.3; malic acid, instead of decreasing, increased by 68 m.eq. Citric acid formation was thus a little greater than in the control leaves cultured on magnesium chloride, and the supply of malic acid was sufficient to bring about accumulation of this substance. The analytical method (2, 21) employed does not distinguish between the enantiomorphs of malic acid. It is quite possible, therefore, that much of the malic acid which accumulated in this sample was D-malic acid which would presumably not enter into the enzymatic reactions.

The intensity of the metabolic activity is emphasized by the increase in unknown acids and also by the increase of 6.3 m.eq. of oxalic acid. Little is known of the metabolic function of oxalic acid in leaves but there is a tendency to regard it as an end-product of oxidative processes. The changes in oxalic acid in most of the tests were small and of doubtful significance, but culture on malic acid is a case in which the increase is almost certainly beyond the experimental error; it amounted to 30 per cent of the oxalic acid present at the start. The picture is one of stimulation of organic acid metabolism, although the enzyme systems were apparently unable to deal with all of the malic acid that entered the leaves. A rough measure of this quantity may be derived from the increase in ash. If this is assumed to represent potassium carbonate, it was the equivalent of 10.8 gm. of malic acid. The increase in total acids as determined was equivalent to 9.5 gm. of malic acid.

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[2] The assistance of Dr. C. I. Bliss in carrying out this calculation is gratefully acknowledged.

The Rosenberg ammonia leaves cultured on DL-malic acid acquired 182 m.eq. of organic acid. The increase in citric acid was 80.4 m.eq., an evidence of intensely stimulated synthesis in these leaves. Malic acid accumulated to the extent of 142 m.eq., and it may be inferred that so much malic acid was taken up that not all of the L enantiomorph was metabolized. Furthermore, there was a definite increase in oxalic acid, again of the order of 30 per cent of the quantity originally present. The unknown acids, however, scarcely changed in amount.

Citrate—The *rustica* nitrate leaves cultured on potassium citrate acquired 171 m.eq. of total acid, equivalent to 10.9 gm. of citric acid. Estimated from the increase in ash, the quantity is 12.0 gm. The actual citric acid content increased by 84.8 m.eq. (5.4 gm.). Malic acid did not decrease; rather it *increased* by 90 m.eq., an evidence of the reversal of the normal direction of the enzymatic reactions of the leaves under the influence of the influx of citric acid. Oxalic acid did not change significantly and the unknown acids diminished slightly.

In the *rustica* ammonia leaves, there was a slightly larger increase in total acids, but the increases in citric and malic acids were a little smaller. The general stimulation of the metabolism brought about a large increase in unknown acids which amounted to 45.1 m.eq.

The effect of culture on ammonium citrate was qualitatively similar to that on potassium citrate although the quantities involved were smaller. Citrate ion was taken up from ammonium citrate solution in smaller amounts, as judged by the increase in total acidity.

These experiments demonstrate that the normal conversion of malic to citric acid observed in tobacco leaves cultured in water in darkness is reversible if citrate ion is supplied from without in sufficient amounts.

Succinate—Tests of the behavior of succinic acid were carried out with all four lots of leaves. Inasmuch as succinic acid is a normal component of the tobacco leaf, although present in only small proportions, it might be anticipated that the introduction of the succinate ion would stimulate the formation of citric acid under the conditions of the experiment. The general effects were similar in all four tests; the average increase in total acids was 166 m.eq. (9.8 gm. calculated as succinic acid). Citric acid increased by almost the same amount in all four, the average being 49.1 m.eq. (3.1 gm.), and, as in the case of culture on citric acid, malic acid also increased, the average being 74.6 m.eq. (5.0 gm.). The completeness with which the added succinic acid was metabolized is shown by the rather small increase in unknown acids in comparison with the quantity of succinic acid introduced. Furthermore, there was a small but definite increase in oxalic acid in the Rosenberg leaves. Direct determinations of succinic acid were not made, so that there is no evidence as to whether succinic acid accumulated at all or was completely metabolized. The data show, however, that succinic acid is an extremely active metabolite. It fits readily into the general scheme of organic acid metabolism and is as effective as citric acid in increasing the malic acid concentration.

Fumarate—Fumaric acid has been claimed to be a normal component of tobacco leaves although the evidence is not entirely convincing. If present at all, the quantity must be small; traces may indeed be encountered when the organic acids from tobacco leaves are subjected to esterification and distillation (22) but it is difficult to prove that the substance found was not produced during the manipulations. The present culture tests with *rustica* leaves, however, gave results that are closely similar to those obtained with succinic acid, save that oxalic acid was probably not increased. Both malic and citric acids increased markedly and there was only a moderate increase in unknown acids, so that fumaric acid did not accumulate to any notable extent. Fumaric acid clearly fits into the metabolic scheme as well as succinic acid and, whether or not it is a normal component present in detectable quantities, enzyme systems capable of transforming it are obviously present.

Pyruvate—The effect of pyruvate was tested with all four lots of leaves with moderately consistent results. Citric acid formation was stimulated in both lots of nitrate leaves to about the same extent as it was by succinate; however, pyruvate was much less effective than succinate in stimulating citric acid formation in the ammonia leaves. Malic acid diminished in the Rosenberg nitrate leaves and in the *rustica* ammonia leaves to about the same extent as it did in the magnesium chloride control; it did not change in the Rosenberg ammonia leaves and decreased scarcely appreciably in the *rustica* nitrate leaves. In all cases, the increase in unknown acids was definitely less than the increase in total acidity and, in the *rustica* nitrate leaves, this increase was considerably less. Accordingly, it appears probable that the newly acquired pyruvic acid was metabolized to some extent in all four lots of leaves and there can be no doubt in the case of the *rustica* nitrate leaves. Pyruvate was sluggishly taken up in comparison to some of the other acids and its effects were, in general, less striking than were those of succinate. Nevertheless, it seems to have entered into the metabolism, for the formation of citric acid was stimulated and, in two cases, malic acid did not decrease, as would be expected from the behavior of the controls.

Lactate—The effect of lactic acid[3] was tested with *rustica* leaves. The increase in total acidity and in ash was about the same as with fumaric acid and the increase in citric acid was also similar to that observed with fumaric or succinic acid and was materially greater than in the control on magnesium chloride. In both lots of leaves, malic acid, instead of decreasing, increased significantly although not strikingly. However, there was a large increase in unknown acids, which suggests that a considerable part of the lactic acid taken in was not metabolized. Because of the crude nature of the preparation of lactic acid used, no inference can be drawn save that lactic acid probably shares to some extent in the general course of the metabolism; enzyme systems that are capable of transforming it are evidently present.

Malonate—The effect of malonate was tested with *rustica* leaves. The salt was taken up in appreciable quantities but the most striking feature of the data is that the unknown acids increased even more than the total acidity. This suggests, in the absence of direct determinations of malonic acid in the tissues, that this substance was not metabolized at all but merely accumulated. However, there was an increase of citric acid in the nitrate leaves which amounted to about one-third the quantity observed in the control, so that the phase of the enzymatic reactions which leads to synthesis of citric acid was not entirely inhibited. The loss of malic acid was roughly twice that in the control and was much greater than would be needed to account for the citric acid produced on any simple hypothesis of transformation. The surplus of unknown acids over the quantity of malonic acid presumably absorbed suggests that part of the malic acid may have had an unusual metabolic fate.

In the ammonia leaves, there was no detectable change in citric acid, and malic acid diminished by a quantity equal to that in the control cultured on magnesium chloride. There was an increase in unknown acids greater than could be accounted for by the absorption of malonic acid. The general impression given by the data is that malonic acid does indeed interfere with the normal course of events when added in relatively large quantity. However, far more detailed analysis of the tissues than was attempted would be required to define the effect.

L(*dextro*)-*Tartrate*—The Rosenberg leaves cultured on tartrate increased moderately in total acidity, citric acid increased to about the same extent as it did in the controls, and malic acid decreased normally in the nitrate leaves. In the ammonia leaves, malic acid did not change; in both lots, however, the unknown acids increased so greatly as to render it probable that tartaric acid was not metabolized to any significant extent. If enzymes are present that bring about chemical change in tartaric acid, there was no evidence that either citric or malic acid was produced from this source. In neither test was there evidence that tartaric acid interfered with the normal course of the metabolism.

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[3] The preparation of lactic acid used was a commercial product that was doubtless heavily contaminated with anhydride and lactide (23) but nevertheless contained a reasonable proportion of L-lactic acid.

Fumarate and Malonate—The effect of an equimolar mixture of fumaric and malonic acid was tested with all four types of leaves to obtain further evidence on the possibility of interference on the part of malonic acid with the course of the metabolism. Malonic acid is known to inhibit the action of succinic dehydrogenase in animal tissues (24). If fumaric acid, the product of the reaction of succinic dehydrogenase upon succinic acid, were absorbed by tobacco leaves along with malonic acid, there should be no great difference in the result from that observed when succinic acid alone was administered. Tests with fumarate, succinate, and malonate separately were carried out with the *rustica* leaves and it will be recalled that both fumarate and succinate gave rise to marked increases in both citric and malic acids. When the mixture of malonate and fumarate was employed, the total acidity of the *rustica* nitrate leaves increased more than in any of the other tests; citric acid increased significantly, although to a smaller extent than with fumarate or succinate alone. Malic acid, however, decreased nearly as much as it did in the magnesium chloride controls instead of increasing as it did with fumarate and succinate. The unknown acids increased nearly as much as the total acidity and far more than can be accounted for on the assumption that half of the acid acquired was malonic acid which remained unchanged in the tissues. The general effects, then, were to increase citric acid appreciably more than in the control although less than in the test with fumarate alone, to decrease malic about as in the control, and to stimulate the production of unknown acids just as malonic acid alone appeared to do.

The effect on the *rustica* ammonia leaves was similar although less pronounced.

The Rosenberg nitrate leaves behaved in much the same way; the increase in citric acid was greater than in the water or magnesium chloride control and about the same as that in the test with succinate; the decrease in malic acid was greater than in the controls. In this case, the apparent transformation of malic to citric acid was promoted. Again, the unknown acids increased much more than could be attributed to the accumulation of malonic acid.

The Rosenberg ammonia leaves also showed evidence of an active metabolism, although at a lower level than in the nitrate leaves. Citric acid increased markedly and malic acid also increased to some extent. Apparently sufficient fumaric acid was taken up to provide for increases in both of these components and there was also evidence of increase in unknown acids if it is assumed that half of the total uptake of acid was malonate which remained unchanged in the leaves.

The results, then, suggest that the malonate introduced along with the fumaric acid did not seriously interfere with the enzyme systems that bring about the formation of citric acid. The nature of the effects upon the metabolism of malic acid remain, however, for future study.

DISCUSSION

The present experiments indicate that the cells of tobacco leaves contain enzyme systems capable of metabolizing most of the organic acids which figure in the Krebs tricarboxylic acid cycle. Lactic acid also appears to be metabolized fairly readily but tartrate and malonate are not. Pyruvate seems to occupy a somewhat special position although this may be apparent only, inasmuch as pyruvate was the least readily absorbed of the acids tested. There is little question that it did enter into the reactions of the cells to some extent.

d-Isocitric acid was not available at the time these experiments were carried out, so that a test with this substance was not made; such a test will be reported on in a later paper.

The evidence is entirely analytical and therefore indirect. Nevertheless, if a certain chemical change occurs in a living tissue, the inference is clear that an enzyme system essential for this change must be present. Where the change is similar to one well known to take place in the more fully investigated animal tissues, similarity in the function of the enzyme system is also to be inferred. However, the similarity probably extends only to the function, for it is extremely unlikely that the specific enzymes of the tobacco leaf are identical with those that bring about similar reactions in such a tissue as pigeon breast muscle. The analogy with the differences between the proteolytic enzymes of plant and of animal tissues may perhaps be pointed out; papain, a typical proteolytic enzyme from a plant, differs in composition and structure as well as in the details of its mode of action from trypsin; yet both enzymes bring about the hydrolysis of peptide bonds in intact proteins.

Although all of the individual members of the Krebs tricarboxylic acid cycle that were tested appeared to fit smoothly into the metabolic system of the tobacco leaf and stimulated at least one of the chemical changes that has been recognized to occur normally, it does not necessarily follow that the Krebs cycle defines the exact sequence of reactions that takes place. Before this can be established, isolation of some, at least, of the specific enzyme systems and reproduction of the reactions *in vitro* will be necessary, doubtless a long and difficult task. For the present, all that it seems safe to assume is that the cells of the tobacco leaf contain systems of enzymes which bring about interconversions of organic acids in a manner that has close analogies with the reactions of the Krebs cycle.

To what extent the conversion of malic into citric acid in the tobacco leaf represents reactions involved in the function of respiration is also uncertain. That respiration was taking place at a moderately high rate is evident from the behavior of the carbohydrate of the leaves. The data for loss of soluble carbohydrate from each of the samples are given in Tables IV and V. Carbohydrates were drawn upon more heavily in the ammonia leaves than in the nitrate leaves, a fact possibly connected with the lower concentration of organic acids in the ammonia leaves, for organic acids themselves appear to be utilized during the respiration of leaf tissues (25). Carbohydrate loss was also high in the leaves cultured on ammonium sulfate and ammonium citrate. However, malonate did not interfere detectably with the utilization of carbohydrate; on the contrary, the loss of carbohydrate from the Rosenberg leaves cultured on the mixture of malonate and fumarate was greater than in most of the other instances, although this was not so for the *rustica* leaves. On the whole, the evidence does not suggest any striking effect of the presence of organic acids in the culture solutions upon the utilization of carbohydrate.

SUMMARY

Detached leaves of the tobacco plant, when subjected to culture in water or in 0.2 M solutions of inorganic salts or of the potassium salts of organic acids, show, by the changes in the content of oxalic, citric, and malic acid and in the total organic acidity, that metabolic reactions continue actively for at least 2 days. When cultured on water or inorganic salts, the main effect is a decrease in the malic acid and the formation of additional citric acid, there being little change in total acidity. The evidence suggests that malic acid undergoes transformations that result in the synthesis of citric acid. The advent of organic acid anions from the culture solution alters the general course of events; culture on citrate reverses the effect of culture on water and this is, in part, true also for succinate and fumarate inasmuch as both citric and malic acids increase. Lactate and pyruvate show a similar though less pronounced effect. Malonate interferes to some extent with the normal transformations but, if furnished together with fumarate, the formation of citrate is stimulated. Tartrate, however, does not appear to have any influence upon the reactions; the increase in unknown organic acids indicates that it merely accumulates.

The general results suggest that those organic acids which figure in the Krebs tricarboxylic acid cycle enter readily into the organic acid metabolism of the tobacco leaf and the inference is drawn that enzyme systems

are present which are capable of metabolizing these acids with considerable rapidity. However, it cannot be concluded that the enzyme systems are, in fact, identical with those characteristic of the Krebs cycle, for no evidence has been secured upon the sequence of the individual reactions. Under the present experimental conditions, only the beginning and the end of the metabolic process are accessible to analysis. Nevertheless, it seems to be clear that the organic acids of the tobacco leaf are members of a dynamic system, the functions of which bear close analogy to those of the systems present in the more thoroughly investigated animal tissues.

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METABOLISM OF 3,5-DIKETOHEXANOIC ACID AND ITS δ-LACTONE BY TISSUE HOMOGENATES

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As an approach to the problem of the possible intermediates in fatty acid oxidation Breusch and Ulusoy (1) studied the metabolism of the δ-lactone of 3,5-diketohexanoic acid (triacetic acid lactone). These workers reported the formation of 1.3 moles of acetoacetate per mole of substrate utilized by minced cat liver and found that the acid remained practically unattacked in kidney, muscle, and lung.

An investigation of this problem was carried out by using a spectrophotometric procedure based upon the high ultraviolet absorption of the polyketo acids (2). Chemical and spectrophotometric evidence indicates that the δ-lactone of 3,5-diketohexanoic acid is enzymatically opened to yield the free 3,5-diketo acid, which is subsequently hydrolyzed to acetoacetic acid and a volatile acid, presumably acetic acid. Both the liver and kidney of the rat catalyze these two consecutive enzymatic processes. The lactonase is more active in kidney than in liver homogenates, and the hydrolysis of 3,5-diketohexanoic acid is more rapid in liver. The pH optima of the two steps are different, suggesting that they are catalyzed by different enzymes. This was proved by physical separation of the two enzyme activities. Neither the lactone nor the free diketo acid is destroyed at an appreciable rate by homogenates of pancreas, spleen, muscle, or brain.

When the degradation of the δ-lactone of 3,5-diketohexanoic acid was studied spectrophotometrically (2), the following observations were made: (*a*) Incubation of the lactone with liver homogenates at pH 7 resulted in a progressive decrease in absorption of the substrate at all wave-lengths, with maxima at 285 and 275 mμ in acid and alkali, respectively. (*b*) Incubation of the lactone with liver or kidney homogenates at pH 5.5 led to a progressive decrease in absorption determined in acid, while a new spectrum appeared in alkali with a maximum at 295 mμ. (*c*) The new spectrum disappeared slowly at pH 5.5, but if the mixture was adjusted to pH 7.0 to 7.4, it disappeared more rapidly. The new spectrum was considered to represent an intermediate in the conversion of the lactone to acetoacetate. The possibility that the intermediate was free 3,5-diketohexanoic acid was suggested by (*a*) the similarity of the ultraviolet absorption curves of the intermediate and certain compounds possessing the grouping

—COCH$_2$CO— (3–5) with respect to absorption maxima and the reversible effect of acid and alkali on the absorption, (*b*) the fact that after acid distillation of mixtures of the lactone and liver or kidney homogenates incubated at pH 5.5 the distillate possessed an ultraviolet absorption curve similar to acetylacetone and formed a red complex with iron, and (*c*) the similarity of the enzymatic and chemical behavior of 3,5-diketohexanoic acid with that of the intermediate compound.

EXPERIMENTAL

Materials—Dehydroacetic acid was prepared by heating gaseous ethyl acetoacetate in an iron pipe at about 450° as described by Collie (6). The product was purified by distillation and crystallization from water and from ether. M.p. 108° (uncorrected).

Analysis—$C_8H_8O_4$. Calculated, C 57.16, H 4.79; found, C 57.29, H 4.99

The δ-lactone of 3,5-diketohexanoic acid was prepared from the above compound as described by Collie (6). M.p. 187° (uncorrected).

Analysis—$C_6H_6O_3$. Calculated, C 57.15, H 4.80; found, C 57.12, H 5.05

Ethyl 3,5-diketohexanoate was obtained by heating the δ-lactone of 3,5-diketohexanoic acid with 3 moles of absolute alcohol in a sealed tube at 110° for 48 hours and purified through the copper salt as described by Sproxton (7).

Analysis—$C_8H_{12}O_4$. Calculated, C 55.81, H 7.02; found, C 55.36, H 7.11

The free acid was prepared by saponification of the ester with the theoretical amount of sodium hydroxide. The alcohol was removed by vacuum distillation. Similar enzymatic data were obtained when the alcohol was not removed.

Acetylacetone, obtained from the Eastman Kodak Company, was redistilled before use. Solutions of sodium acetoacetate were prepared as described by Davies (8).

Methods—Male and female rats of the Buffalo strain, weighing 150 to 350 gm. and fed *ad libitum*, were employed. The animals were killed by decapitation followed by exsanguination and the fresh tissues immediately ground with 1.5 volumes of cold water in the Potter-Elvehjem homogenizer (9). The homogenates were incubated with an equal volume of 0.025 M substrate in 0.13 M phosphate buffer at 37°. Aliquots were removed at suitable intervals for spectrophotometric and chemical analysis. Controls with substrate alone and homogenate alone were employed. The substrates were stable under the conditions of incubation employed.

Ultraviolet absorption studies were carried out with the Beckman model DU spectrophotometer with a 1 cm. light path. The absorption curves in acid and in alkali for 3,5-diketohexanoic acid lactone and the free diketo acid are given in Fig. 1. The relationship between degree of enolization and absorption of the diketo acid is similar to that observed with 2,4-diketo acids (3). The procedure used for following the enzymatic degradation of 3,5-diketohexanoic acid lactone was as follows: A 0.4 cc. aliquot was treated with 19.6 cc. of 3 per cent perchloric acid (10) and the mixture filtered. Aliquots of the clear filtrate were treated with an equal volume of 0.5 N sodium hydroxide or 0.1 N hydrochloric acid and read against a homogenate blank which was treated in identical fashion, except that substrate was omitted. A similar procedure was employed for following the disappearance of 3,5-diketohexanoic acid, except that the final dilution was 2.5 times greater. The values of absorption are expressed as *D*-Alk or *D*-Ac for the data obtained in sodium hydroxide and hydrochloric acid, respectively. Wave-lengths are given in parentheses.

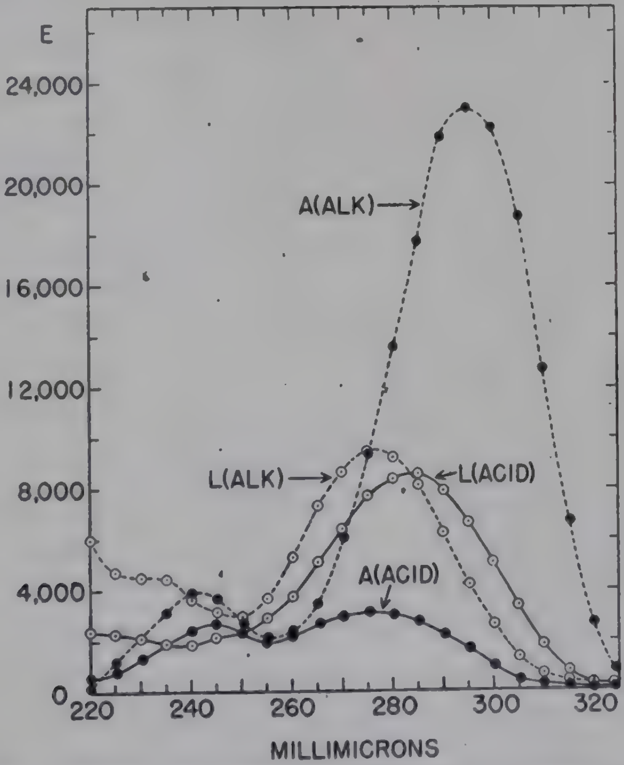


FIG. 1. Ultraviolet absorption spectra of 3,5-diketohexanoic acid (*A*) and the δ-lactone of 3,5-diketohexanoic acid (*L*); free acid, ●; lactone, ○; continuous curves, in 0.1 N hydrochloric acid; dash curves, in 0.1 N sodium hydroxide. Concentration, 5×10^{-5} M (free acid) and 1.25×10^{-4} M (lactone).

The aniline citrate method of Edson (11) was employed in certain experiments. With this procedure acetoacetate and 3,5-diketohexanoate were completely decarboxylated, yielding theoretical amounts of carbon dioxide, whereas the more stable lactone liberated negligible amounts of carbon dioxide in 60 minutes at 37°.

Determinations of total ketone, acetone, and acetylacetone were carried out on distillates of 1 to 4 cc. aliquots of the incubated mixtures acidified with 2 cc. of 0.67 N sulfuric acid. A quantitative colorimetric method for acetylacetone based upon the red color of the iron-acetylacetone complex (12) was developed. A titrimetric procedure for the determination of acetylacetone and other β-dicarbonyl compounds by use of the copper-acetylacetone complex has been described (13). The present method has the advantage of being relatively simple, and when applied to distillates eliminates interference by non-volatile compounds such as 2,4-diketo acids. Since acetone and other methyl ketones give no color with ferric chloride, acetylacetone can be determined in acid distillates of mixtures containing acetoacetic acid and other β-keto acids. The following procedure was employed: A 5 cc. aliquot of the distillate containing between 0.5 and 5 μM of acetylacetone was added to 1 cc. of freshly prepared 0.04 M ferric chloride in a colorimeter tube and mixed thoroughly. Maximum color development was observed in 5 minutes, at which time the tubes were read in the Coleman spectrophotometer at 500 mμ against a blank containing 5 cc. of water and 1 cc. of ferric chloride. The color intensity decreased 5 per cent in 1 hour. The optical density was directly proportional to acetylacetone concentration. Recovery of acetylacetone added to homogenates was between 98 and 101 per cent in the presence or absence of 1000 times as much acetone. Duplicate determinations checked within 2 per cent. Distillates of homogenate blanks gave no color. In the present experiments the pH of the distillate plus ferric chloride was always between 2.4 and 2.5. A decrease in complex formation occurs if the pH is increased to 3.0. In cases in which the pH of the solution to be analyzed is variable, a strongly buffered system would therefore be required. The method could be adapted to the determination of smaller amounts of acetylacetone by developing the color in a smaller volume.

Acetylacetone was also determined from the ultraviolet absorption of the distillates in 0.1 N sodium hydroxide at 295 mμ by employing a molar extinction coefficient (E) of 20,000. The absorption of distillates of controls containing homogenate alone was negligible under these conditions. The values obtained by this procedure were in good agreement with those determined by the colorimetric procedure (Figs. 2 and 3). Differences of less than 6 per cent were found between the data obtained by the two methods. In confirmation of Breusch and Ulusoy (1) we have noted that during distillation about 5 per cent of the lactone of 3,5-diketohexanoic acid decomposes, yielding acetylacetone. The total ketone and acetylacetone values were corrected by subtracting this value which was always quite small. In contrast to the stable lactone, the free 3,5-diketo acid could be quantitatively accounted for as acetylacetone on acid distillation. No diketo acid or acetylacetone was found in the residue of the distillation flask and the distillate yielded no carbon dioxide when tested by the aniline citrate procedure.

Acetone was determined by the method of Greenberg and Lester (14). The colorimetric readings were made at 440 mμ. Under these conditions acetylacetone gives about 8 per cent as much color as does acetone. The values were therefore corrected according to the concentration of acetylacetone determined as described above. Total ketone was determined by the method of Behre (15), in which acetylacetone and acetone give equivalent amounts of color. Acetone values obtained by subtracting acetylacetone from total ketone values agreed closely with those obtained by the procedure of Greenberg and Lester, except in cases in which small amounts of acetone were determined.

Results

Degradation of 3,5-Diketohexanoic Acid Lactone by Liver and Kidney Homogenates

The data given in Fig. 2 summarize the chemical and spectrophotometric data obtained when the δ-lactone of 3,5-diketohexanoic acid was incubated with rat liver homogenates at pH 5.5 and 7.2. At pH 7.2 no evidence of an intermediate was obtained. The absorption in acid at 285 mμ (*D*-Ac (285)) and in alkali at 295 mμ (*D*-Alk (295)) decreased progressively with time, and no acetylacetone was found in the distillates. At pH 5.5, however, a more rapid decrease in *D*-Ac (285) occurred, and a sharp rise in *D*-Alk (295) was observed initially, followed by a gradual decrease over a period of 3 hours. The formation of acetylacetone followed a similar pattern. With kidney homogenates (Fig. 3) the disappearance of the absorption in acid at 285 mμ was faster than with liver at pH 5.5 and the absorption of the intermediate was high, indicating a slower rate of disappearance than with liver. At pH 7.1 the *D*-Alk (295) and acetylacetone values were almost equal to those observed with liver incubated at pH 5.5. At pH 5.5 kidney homogenates destroyed the lactone rapidly and the intermediate slowly, leading to an accumulation of intermediate as indicated by high values of *D*-Alk (295) and acetylacetone. Since the absorption in alkali at 295 mμ is due to the lactone as well as the intermediate, an attempt was made to correct the observed *D*-Alk (295) values by subtracting the absorption due to the lactone. The latter value was obtained by assuming that the change in *D*-Ac (285) for a given time interval divided by the total density change represented the fraction of lactone destroyed. Thus the value to be subtracted from the observed *D*-Alk (295) value was obtained by multiplying the fraction of lactone destroyed by the initial *D*-Alk (295) value. (The true correction is actually somewhat smaller than this value, since, as will be described below, free 3,5-diketohexanoic acid has a small absorption in acid at 285 mμ.) As shown in Figs. 2 and 3, the shapes of the curves for acetylacetone and corrected *D*-Alk (295) are quite similar. The ratio of acetylacetone to corrected *D*-Alk (295) was close to 0.4 in all of these experiments.

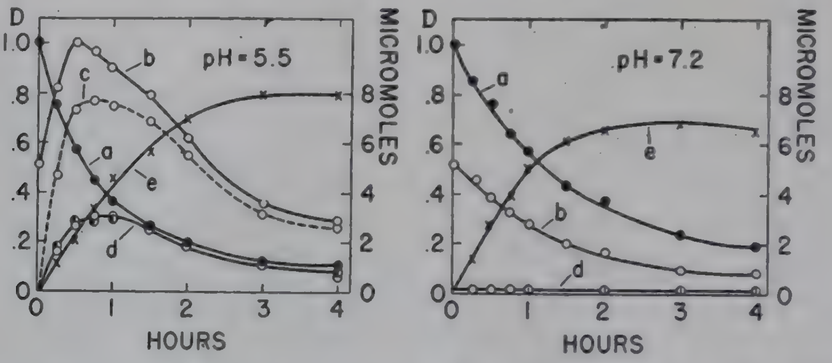


FIG. 2. Incubation of 3,5-diketohexanoic acid lactone with liver homogenate at pH 5.5 and 7.2. Equal volumes of homogenate and 0.025 M lactone in 0.13 M phosphate buffer were mixed and aliquots removed at the indicated intervals for chemical and spectrophotometric analysis. Curve *a*, absorption in acid at 285 mμ; Curve *b*, absorption in alkali at 295 mμ; Curve *c*, absorption in alkali at 295 mμ (corrected as described in the text); Curve *d*, acetylacetone by colorimetric method (○) and ultraviolet absorption (◐); Curve *e*, acetone (total ketone minus acetylacetone). Ketone values expressed as micromoles per 0.8 cc. of reaction mixture ≈ 10 μM of initial substrate.

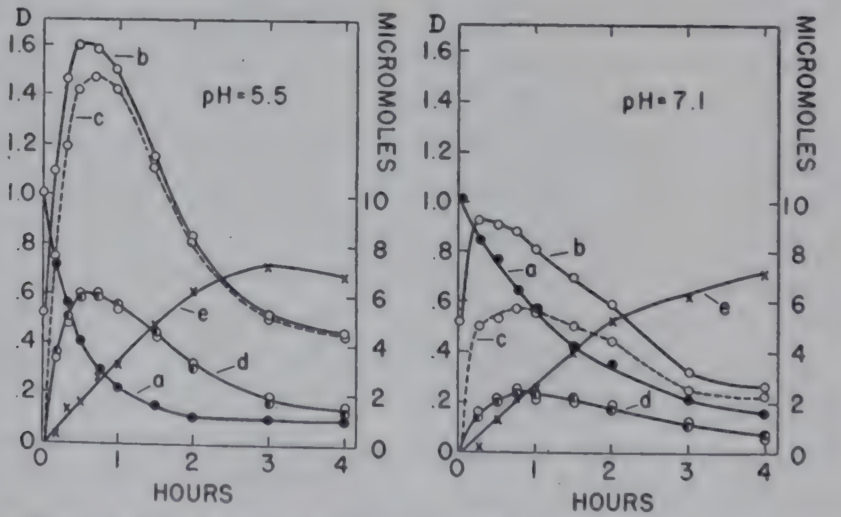


FIG. 3. Incubation of 3,5-diketohexanoic acid lactone with kidney homogenate at pH 5.5 and 7.1. Curves designated as in Fig. 2.

The final result in all four experiments was the formation of 7 to 8 μM of acetone from 10 μM of lactone. Similar results were obtained anaerobically and in a few experiments with rats fasted for 3 to 4 days. Conversion to β-hydroxybutyric acid was ruled out by analyses according to the procedure of Van Slyke (16). Recovery experiments in which 5 μM of acetoacetate were incubated with liver and kidney homogenates under similar conditions revealed that 0.3 to 0.9 μM of acetoacetate disappeared in 1 hour as determined by the Behre and Van Slyke procedures. This small but definite destruction of acetoacetate by liver and kidney homogenates did not occur with boiled homogenates.

In another series of experiments with liver and kidney homogenates at pH 5.5, the pH of the incubated mixture was adjusted to values between 6.5 and 8.0 when the *D*-Alk (295) value was maximal. The subsequent rate of decrease of *D*-Alk (295) was most rapid at about pH 7.

The degradation of the lactone by kidney homogenate at pH 5.5 was also followed by the aniline citrate method. The liberation of carbon dioxide paralleled the total ketone values (Table I). Since in the early part of the reaction most of the ketone formed may be accounted for as acetylacetone, these findings suggested that the intermediate compound underwent decarboxylation catalyzed by aniline citrate.

Studies on 3,5-Diketohexanoic Acid

The 3,5-diketo acid was found to exhibit ultraviolet absorption maxima at 295 and 275 mμ in alkali and acid, respectively (Fig. 1). The corresponding molar extinction coefficients were 23,000 and 3010. Incubation of this compound with homogenates of liver and kidney resulted in a progressive decrease of absorption at all wave-lengths. Under the conditions employed the initial density was 1.15 in alkali at 295 mμ. The final density value was 0.035, indicating a very low absorption of the products of the reaction. Activity values were obtained from the change in *D*-Alk (295) for a given time interval divided by the total change. Distillates of aliquots of the incubated mixtures were analyzed for acetylacetone and acetone. Good agreement was obtained between the disappearance of acetylacetone in the distillates and the decrease in *D*-Alk (295). Approximately 1 mole of acetone was formed per mole of substrate destroyed (Table II). The rate of the reaction was linear throughout the first 80 per cent of its course. Previous experiments indicated that the pH optimum for the disappearance of the intermediate compound in the metabolism of the lactone was about 7. The optimal pH for the disappearance of 3,5-diketohexanoic acid was found to be at about 7 to 7.3 (Fig. 4). The relationship between activity and homogenate concentration is linear over the concentration range studied (Fig. 5). Homogenates of brain, spleen, pancreas, and muscle were incubated with 3,5-diketohexanoic acid at pH 7.3. No change in the absorption curve of the diketo acid was observed after 75 minutes of incubation.

Calculation of Lactonase Activity

As noted above, the observed *D*-Ac (285) values are slightly greate than the true absorption of the lactone, due to the small absorption o

TABLE I

Relation between Ketone Formation and Carbon Dioxide Liberation by Aniline Citrate

Data obtained with kidney homogenate incubated at pH 5.5. Values expresse as micromoles per 0.8 cc. of mixture ≈ 10 μM of initial substrate.

Time	Acetylacetone	Total ketone	Carbon dioxide
min.	μM	μM	μM
6	2.04	2.12	2.25
20	4.40	5.45	5.64
35	5.86	7.95	7.41

TABLE II

Hydrolysis of 3,5-Diketohexanoic Acid by Liver and Kidney Homogenates

Data obtained with 1 cc. of homogenate and 1 cc. of 0.025 M substrate in phospha buffer at pH 7.3.

	Time	Substrate disappearance	Acetylacetone	Acetone
	min.	μM	μM	μM
Liver	10	4.90	20.0	4.60
	20	10.5	15.3	9.71
	30	14.5	9.71	15.2
	45	22.8	2.75	21.8
	60	24.4	1.03	22.4
Kidney	30	7.17	17.9	6.30
	60	14.4	11.3	15.2
	90	21.6	3.68	18.3
	120	23.2	2.50	21.1

3,5-diketohexanoic acid at 285 mμ in acid. However, the concentratio of 3,5-diketohexanoic acid at any point may be obtained from the concentration of acetylacetone. Thus the absorption due to the diketo aci at 285 mμ may be obtained from the following equation, where *AA* is th concentration of acetylacetone in moles per 10 cc. of the incubated mixture and 2580 is the molar extinction coefficient of the diketo acid at 28 mμ in acid:

$$D = AA \times 2580$$

This correction was small at pH 6.3 with liver homogenates and increased as the pH of incubation was decreased. This is illustrated by the pH-activity curve for the disappearance of the lactone (Fig. 4), in which both the observed and corrected ΔD values are given. The pH optimum was at 5.9 to 6.3 for both liver and kidney homogenates.

When the D-Ac (285) values given in Figs. 2 and 3 were corrected as described above, the disappearance of the lactone was found to follow a first order reaction. The rate constants for liver homogenates, calculated from the equation $k = 1/t \log 100/100 - x$, where t is expressed in minutes and x represents the per cent substrate destroyed, were 0.0122 and 0.00459 at pH 5.5 and 7.2, respectively. The corresponding constants for kidney homogenates were 0.0224 and 0.00581, at pH 5.5 and 7.1. The relation between liver homogenate concentration and activity at pH 6.3 is given in Fig. 5.

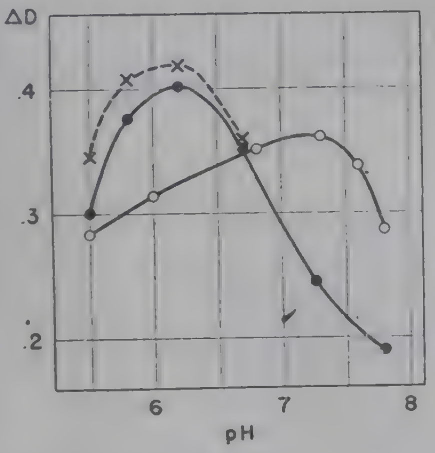


FIG. 4

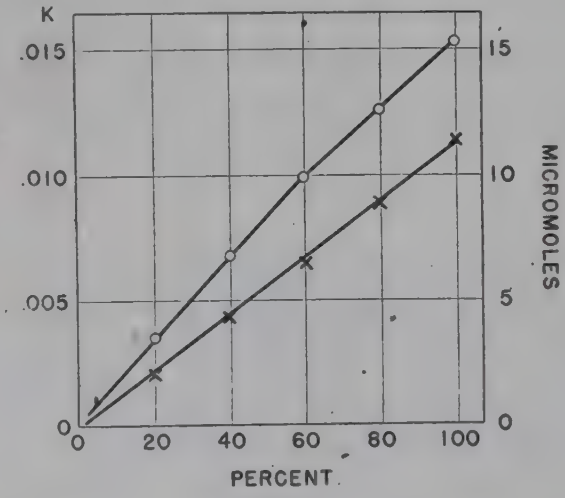


FIG. 5

FIG. 4. pH-activity curves for the disappearance of the lactone (●) and free 3,5-diketohexanoic acid (○). × represents values of ΔD corrected as described in the text. Values expressed as change in absorption in acid at 285 mμ and absorption in alkali at 295 mμ for the lactone and free acid, respectively, in 15 minutes.

FIG. 5. Effect of homogenate dilution on activity. Abscissa, per cent of initial homogenate concentration. Ordinate (right-hand), disappearance of 3,5-diketohexanoic acid (×) in terms of micromoles per cc. of homogenate or diluted homogenate per 20 minutes at pH 7.3. Ordinate (left-hand), disappearance of the lactone (○) expressed in terms of the rate constant, k, determined at pH 6.3.

Homogenates of muscle, brain, pancreas, spleen, testis, and stomach were incubated with the lactone at pH 6.3. After 2 hours the absorption of the substrate decreased only 3 to 5 per cent, except in the case of brain where 10 to 15 per cent of the absorption disappeared.

Liver and kidney homogenates were incubated with dehydroacetic acid at pH 6.3. This compound has ultraviolet absorption maxima at 290 and

305 mμ in alkali and acid, respectively, with corresponding molar extinction coefficients of 9700 and 13,800. No decrease in absorption was observed after incubation for 140 minutes.

Separation of Two Enzymatic Activities

By means of high speed centrifugation a fairly good separation of the lactonase from the enzyme acting upon the diketo acid was obtained. As shown in Table III, about 70 per cent of the lactonase activity was found in the pellet, while about 85 per cent of the diketo acid enzyme remained in the clear supernatant. An 8-fold purification of the latter enzyme was obtained as follows: Alcohol was added to the supernatant at −5° to a final concentration of 20 per cent. After standing at −5° for 12 hours, the precipitate was removed by centrifugation at −5°, and the alcohol concentration increased to 50 per cent. The mixture was allowed to stand at −20° for 12 hours and the precipitate was collected by centrifugation and was dissolved in cold water. The activity values for the initial homogenate and partially purified preparation were 0.76 and 6.2 μM of diketo acid hydrolyzed per mg. of nitrogen per 20 minutes. This preparation was not active against the lactone and yielded 1 mole of acetone per mole of diketo acid metabolized. The acetone formed was identified by isolation as the 2,4-dinitrophenylhydrazone. After a single recrystallization from water the melting point was 127° (uncorrected) and a mixed melting point with an authentic sample of acetonedinitrophenylhydrazone was 127° (uncorrected).

TABLE III

Separation of Lactonase from Enzyme Hydrolyzing Free 3,5-Diketohexanoic Acid

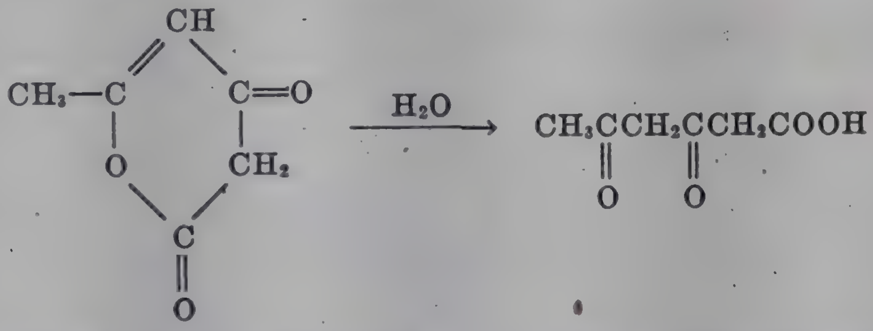
10 cc. of liver homogenate were centrifuged at 18,000*g* at 5° for 2 hours. The pellet was suspended in 10 cc. of water. Hydrolysis of 3,5-diketohexanoic acid is expressed as the total micromoles of substrate destroyed in 20 minutes for each fraction. The first order constants for the disappearance of the lactone were multiplied by the volume (cc.) of each fraction.

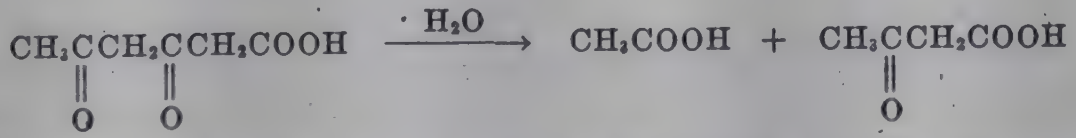
Experiment No.	Substrate	Original homogenate	Pellet	Supernatant
1	Diketo acid	98.1	5.16	88.6
	Lactone	0.185	0.133	0.036
2	Diketo acid	101.0	23.2	81.0
	Lactone	0.179	0.128	0.039

Experiments with larger amounts of substrate were carried out in an attempt to demonstrate significant amounts of volatile acid in the distillates. The following procedure was employed: 262 μM of 3,5-diketohexanoic acid were incubated at pH 7.3 with 8 cc. of the supernatant obtained by high speed centrifugation. The ultraviolet absorption, acetone, and volatile acid formed (17) were determined after 30 minutes of incubation. The values of substrate disappearance, acetone, and volatile acid were 120, 118, and 105 μM, respectively. It is assumed, although not proved, that this acid is acetic acid.

DISCUSSION

The present results indicate that the metabolism of the δ-lactone of 3,5-diketohexanoic acid involves a two-step enzymatic mechanism whereby the lactone is first opened to yield 3,5-diketohexanoic acid, as shown in the accompanying diagram. The evidence is compatible with the hypothesis that the latter compound is hydrolyzed to acetoacetic and acetic acids.





$$CH_3\underset{\overset{\|}{O}}{C}CH_2\underset{\overset{\|}{O}}{C}CH_2COOH \xrightarrow{H_2O} CH_3COOH + CH_3\underset{\overset{\|}{O}}{C}CH_2COOH$$

The finding of more than 1 mole of acetoacetate formed per mole of lactone metabolized (1) has not been confirmed for rat liver homogenates.[1] In contrast to the kidney of the cat, reported to be inactive towards the lactone, rat kidney homogenates exhibit considerable lactonase activity. The present studies indicate that 3,5-diketohexanoic acid is hydrolyzed by an enzyme not associated with the sedimentable fraction of rat liver. This is of interest in view of the fact that Lehninger's fatty acid oxidase system is apparently found in the sedimentable fraction (18, 19) and suggests that fatty acid oxidation in this system may not necessarily go

[1] Breusch and Ulusoy found that the molar ratio of acetone formation (determined by precipitation with 2,4-dinitrophenylhydrazine) to lactone disappearance was 1.29. However, the values for "acetone" by their colorimetric procedure were about 1.7 times greater than those obtained by precipitation. In the light of the present findings it seems probable that the extra color formation was due to acetylacetone and is compatible with the formation of significant amounts of free 3,5-diketohexanoic acid. The presence of 3,5-diketohexanoic acid would, of course, complicate calculation of the ratio of acetoacetate formation to diketo acid breakdown.

through a diketo acid stage.[2] Further studies are necessary to elucidate the metabolic significance of this enzyme as well as that of the lactonase. The latter enzyme does not act upon dehydroacetic acid under conditions in which the lactone of 3,5-diketohexanoic acid is rapidly metabolized.

The hydrolysis of 3,5-diketohexanoic acid to acetoacetic and acetic acids appears to be analogous to the hydrolysis of 2,4-diketo acids which yields pyruvic and fatty acids in that both reactions involve hydrolysis of a C-acyl group (3). Furthermore, both activities remain in the supernatant after high speed centrifugation of liver homogenates. The possibility that the same enzyme hydrolyzes 2,4-diketo and 3,5-diketo acids must be considered. Work along these lines is in progress.

It is a pleasure to thank Dr. Jesse P. Greenstein for his valuable advice.

SUMMARY

Aqueous homogenates of rat liver and kidney catalyze the hydrolysis of the δ-lactone of 3,5-diketohexanoic acid, yielding free 3,5-diketohexanoic acid. This reaction occurs more rapidly in kidney than in liver and has a pH optimum at 5.9 to 6.3. Neither liver nor kidney metabolizes dehydroacetic acid. Free 3,5-diketohexanoic acid is hydrolyzed by kidney and liver homogenates to yield 1 mole each of acetoacetic acid and a volatile acid, probably acetic acid. This reaction is faster in liver and has a pH optimum at 7 to 7.3. The two enzymes have been separated by high speed centrifugation of liver homogenate. The lactonase is associated with the pellet, while the enzyme hydrolyzing 3,5-diketohexanoic acid remains in the supernatant. A partially purified enzyme preparation possessing activity only towards the diketo acid has been prepared from rat liver.

A spectrophotometric procedure based upon the high ultraviolet absorption of polyketo acids was employed in the course of these studies. A simple colorimetric method for the determination of acetylacetone was described.

Addendum—After this paper was submitted, a very interesting report on the metabolism of triacetic acid by Witter and Stotz appeared (20). The results of this study and the present data are in essential agreement with respect to the metabolism of triacetic acid. These workers reported that triacetic acid lactone was metabolized by rat liver at about one-eighth the rate of the free acid. The present studies indicate that under optimal conditions the lactone and free acid are metabolized at rates of about the same order of magnitude by liver and that the lactone is metabolized more rapidly than the free acid by kidney. The difference between the present results and those of Witter and Stotz may be due to the fact that the pH optima for the metabolism of the free acid and of the lactone are markedly different (*cf.* Fig. 4 of the present communication).

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[2] Under conditions in which n-hexanoic, β-ketohexanoic, and 2,4-hexadienoic acids were oxidized rapidly by a sedimentable fraction of rat liver fortified as described by Lehninger, both 2,4- and 3,5-diketohexanoic acids were metabolized at negligible rates. This suggests that these polyketo acids as such are not intermediates in the oxidation of n-hexanoic acid in this system, but are probably involved in other metabolic pathways.

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THE β-CARBOXYLASES OF PLANTS

II. THE DISTRIBUTION OF OXALACETIC CARBOXYLASE IN PLANT TISSUES*

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In a previous survey of the possible occurrence of oxalacetic (OAA) carboxylases in plant tissues, detection of the enzyme was found to be complicated by the wide-spread distribution of pyruvic carboxylase, and by the ubiquitous presence of heat-stable catalysts for the decarboxylation of the β-keto acid. Direct demonstration of a heat-labile, β-(de)carboxylase (OAA carboxylase) distinct from an α-(de)carboxylase (pyruvic carboxylase) was, however, achieved in the case of parsley root and of the crystalline globulins of squash and pumpkin seeds (1). Indirect evidence was presented at the same time for the presence of OAA carboxylase in a number of other plant sources (parsnip, radish, carrot, and cabbage).

The OAA carboxylase of parsley root has subsequently been studied in some detail (2, 3). This enzyme catalyzes the Wood-Werkman reaction.

$$\text{Oxalacetate} \rightleftharpoons \text{pyruvate} + CO_2$$

Because this reaction appears to be a main step in the interconversion of carbohydrate and the biologically important dicarboxylic acids, it appeared profitable to reexamine the question of the distribution of similar enzymes in other plant sources.

This paper will summarize the evidence that OAA carboxylase is present in roots, tubers, leaves, and seeds of a wide variety of plant species.

Materials and Methods

Preparation of Materials for Testing[1]—The plant materials were bought on the market. All plant tissues were thoroughly cleaned and ground as previously described (1), except in the case of wheat germ, which was available as a powder. The dry legumes were soaked overnight before grinding. 4 volumes of water or of 0.025 M phosphate buffer, pH 7.4, were stirred for ½ hour with the wheat germ and with the ground seeds; otherwise no water was added. In all cases coarse solids were removed

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[1] The author is indebted to Dr. J. Ceithaml for the information which led to the use of ammonium sulfate precipitates in the procedures described.

by straining the extract or plant juice through cloth, sometimes with the aid of a fruit press. Operations were carried out at room temperature, but the fresh extracts were kept in an ice bath, and it seemed advantageous (though not always necessary) to conduct the centrifugations in a cold room (−4°). The press-juice or extract was then made approximately one-third saturated with ammonium sulfate, either by addition of 0.5 volume of saturated ammonium sulfate (neutralized with NH_4OH) or by direct addition of 25 gm. of solid ammonium sulfate per 100 ml. The first procedure was generally employed. The second procedure was used in only a few cases, but seemed to give equally satisfactory results. The first precipitate was removed by centrifugation and discarded. If a fat layer appeared, it was strained off through glass wool. In almost every case, any pyruvic carboxylase activity and any oxygen consumption which might be exhibited by the original juice were inactivated or removed by this precipitation. The oxalacetic carboxylase activity remaining in solution was then precipitated either by addition of an equal volume of saturated, neutralized ammonium sulfate or by direct addition of 25 gm. of solid ammonium sulfate per 100 ml. The precipitate was centrifuged and taken up in a minimum volume of 0.025 M phosphate buffer, pH 7.4, and dialyzed against the same buffer at 4° until free from ammonium sulfate. The protein solution thus obtained was examined for OAA carboxylase activity. A considerable amount of insoluble material was usually suspended in this final solution. The precipitate could sometimes only be removed by high speed centrifugation (18,000 × g, Servall angle head, at 0°). Such clearing of the solution was not necessary for the assay, but was carried out in order to obtain a determination of the dry weight of soluble protein present. No activity was ever found associated with the insoluble material. Dry weights were obtained by drying in an oven at 110°, and were corrected for the known buffer content of the solution.

Test Procedure—The procedure employed for demonstration of the enzyme was identical with that previously devised for the assay of OAA carboxylase in parsley root, and has been described in detail (3). It can be used only with preparations free from pyruvic carboxylase activity. The rate of CO_2 evolution from OAA added to enzyme is measured by the usual Warburg manometric technique at 30°. The medium contains 0.1 M acetate buffer, pH 5.0, and 0.01 M $MnCl_2$. In the absence of added Mn^{++}, virtually no heat-labile activity was observed in the dialyzed plant preparations. The decarboxylation shows first order kinetics with respect to substrate. This has been true, at least as a first approximation, in the case of all the plant enzymes hitherto tested. The velocity of the decarboxylation is therefore expressed as a first order reaction rate constant, k,

$$k = \frac{2.3}{t_2 - t_1} \log \cdot \frac{(\text{OAA}_1)}{(\text{OAA}_2)}$$

where (OAA_1) and (OAA_2) are the OAA concentrations at times t_1 and t_2 (in minutes). Mn^{++} ions alone, in the absence of enzyme, also exhibit a catalytic effect on the decarboxylation of OAA (3, 4), and this "blank" decarboxylation likewise shows first order kinetics with respect to substrate. Heat-inactivated enzyme may cause a small change in this velocity constant, and blanks were therefore always determined in the presence of heat-inactivated enzyme. The difference between the rate constant, k, observed in the presence of active enzyme and the blank rate constant, k blank, may then serve as a measure of the amount of enzyme present. Under the conditions employed, the blank reaction rate constant was usually about 0.08 min.$^{-1}$. It is necessary to employ sufficient protein in each case so that the difference between rate constants of the unheated sample and of the blank is well outside the limits of experimental error.[2] Previous failure to demonstrate OAA carboxylase in press-juice from sources such as spinach, which showed no pyruvic carboxylase activity, was due to the fact that the differences often observed between CO_2 evolution rates in the presence of unheated and heated press-juice were regarded as too small to be positively indicative of the presence of the enzyme. In the results reported in this paper, a minimum difference in rate constants of 40 per cent of the higher figure was achieved in every instance. A few determinations of the presence of oxalosuccinic carboxylase were likewise carried out under conditions used to demonstrate this enzyme in parsley root (5).

RESULTS AND DISCUSSION

All plant extracts were routinely examined for OAA carboxylase before precipitation with ammonium sulfate. Since these results often gave no clear cut information, they will not be summarized. A considerable body of data of this type has already been published (1). However, typical data obtained with a crude undialyzed wheat germ extract, which was not previously examined, are given in Table I. These may serve as an example of the manner in which pyruvic carboxylase interferes with the assay for OAA carboxylase.

The figures for Vessels 1 and 2 show that the rate of CO_2 evolution from OAA is considerably lower in the presence of a heat-inactivated extract than in the presence of unheated extract. It is further apparent (Vessels 3 and 4) that this difference is enhanced in the presence of added MnCl_2.

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[2] The success of the procedure often depends mainly on the observation of this requirement, hence the necessity in most cases of achieving a protein concentrate from the plant press-juice.

TABLE I

Action of Crude Wheat Germ Extract on Oxalacetate and Pyruvate

Vessel No.	Additions	CO_2 evolution					
		2.5 min.	5 min.	10 min.	15 min.	20 min.	30 min.
		μl.	μl.	μl.	μl.	μl.	μl.
1	180 μl. oxalacetic acid	31	60	113	152	194	238
2	Like (1), but enzyme heat-inactivated	9	20	35	47	67	84
3	180 μl. oxalacetic acid, 0.01 M $MnCl_2$	140	230	324	356	362	364
4	Like (3), but enzyme heat-inactivated	17	38	74	99	122	144
5	224 μl. pyruvate*	38	85	165	212	222	222

All vessels contain 1 ml. of wheat germ extract (made by extracting 1 part of wheat germ with 4 parts of 0.02 M phosphate, pH 7.4). Total volume 2 ml., made up to contain 0.1 M acetate buffer, pH 5.0. All values corrected for small blanks.

* Pyruvic carboxylase is completely inactivated by heating.

TABLE II

Activity of Crude Oxalacetic Carboxylases Prepared from Various Plant Sources

Family	Species	Common name and part of plant used	Activity, $k - k$ blank	Volume concentration factor
			mg. dry weight min.^{-1} per mg.	
Umbelliferae	*Petroselinum hortense* Hoffm.	Parsley root	0.060	2
	Pastinaca sativa L.	Parsnip "	0.040	2
	Daucus carota L.	Carrot "	0.026	6
Cruciferae	*Brassica oleracea* var. *capitata* L.	Cabbage head	0.015	10
	Brassica oleracea var. *botrytis* L.	Cauliflower	0.009	50
	Raphanus sativus L.	Red radish root	0.037	10
Chenopodiaceae	*Spinacia oleracea*	Spinach leaf	0.009	10
	Beta vulgaris var. *crassa* Alef.	Beet root	0.032	5
Leguminosae	*Ervum lens*	Lentil seeds	0.002	20
	Pisum sativum L.	Green peas*	0.002	30
Solanaceae	*Solanum tuberosum*	White potato tubers	0.005	20
Cucurbitaceae	*Cucurbita pepo*	Pumpkin seeds†	0.015	20
	Cucumis sativus L.	Cucumber pulp (parenchyma of mesocarp)	0.043	20
Gramineae	*Triticum sativum*	Wheat germ	0.029	1

All preparations are protein fractions which precipitate at between about one third and two-thirds saturation with $(NH_4)_2SO_4$.

* Vaughan's Laxton's progress peas.

† Vaughan's small sugar pie pumpkin.

The presence of pyruvic carboxylase is shown in Vessel 5. 2 moles of CO_2 are therefore released for each mole of OAA added if the reaction is allowed to proceed to completion, as is the case in Vessel 3. Since the rates of CO_2 evolution in Vessels 1 and 3 are more than twice as great as those in Vessels 2 and 4 respectively, it is apparent that the heat-labile factor attacks OAA directly (*i.e.*, the CO_2 evolution from OAA in the presence of unheated enzyme cannot be attributed to the non-enzymic decarboxylation of OAA, followed by the decarboxylation of pyruvate by pyruvic carboxylase (1)). However, one cannot conclude from such data whether pyruvic carboxylase itself is the enzyme which attacks OAA. It is also impossible to get an accurate measure of the rate of decarboxylation of OAA to pyruvate, since the further decarboxylation of pyruvate occurs simultaneously.

When the wheat germ extract was subjected to the simple fractionation procedure described, a preparation was obtained which contained OAA carboxylase completely free from pyruvic carboxylase. Since it has been possible to achieve such a separation in the case of every plant source examined in which the two enzymes were initially found to be associated, one may conclude that the α-(de)carboxylase and the β-(de)carboxylase are distinct and specific enzymes.

Typical figures for the OAA carboxylase content of protein preparations from fourteen different plant sources are given in Table II. The results are expressed as the quantity of enzyme per mg. of dry weight of the preparation ($k - k$ blank per mg. of dry weight). Since preparations from the several sources vary considerably in activity, it is apparent that different amounts of protein were required to achieve optimal test conditions in the various cases. Sufficient protein was used to make the uncorrected k value about 0.16 min.$^{-1}$, or twice the value of the blank. On the other hand, uncorrected k values which approached 1 min.$^{-1}$ were avoided, since the reaction was then too rapid for accurate measurement. As a guide to a preliminary approximation of the amount of enzyme to employ in the test a "volume concentration factor" for each preparation has been given in the last column of Table II. This is simply the approximate ratio of the initial volume of the plant juice or extract to the final volume of the dialyzed test solution, after appropriate dilution has been made so that 1 ml. is a suitable amount for assay. It may be noted that no volume concentration of the wheat germ extract and little concentration of parsnip and parsley root were required. In most of the other cases considerable, though varying, degrees of concentration were employed. When the protein precipitates were bulky, it was sometimes necessary to reprecipitate the protein with $(NH_4)_2SO_4$ in order to achieve the necessary volume concentration. This was usually necessary with spinach, cauliflower, legumes, and potatoes.

Most of the preparations listed in Table II contained pyruvic carboxylase in the initial juice or extract. The exceptions were spinach, potato, and beets.

Special mention may be made of the results with pumpkin seeds. These seeds are a rich source of the easily crystallizable cucurbit globulins, which exhibit a heat-labile OAA carboxylase activity (1) not activated by divalent cations. In order to exclude this protein, the preparation was dialyzed thoroughly against water and centrifuged until clear. The solution so obtained was then subjected to a repetition of the ammonium sulfate precipitation, the precipitate being dissolved in a minimum volume. Ammonium sulfate was removed by the usual dialysis against phosphate, and the solution was then dialyzed again against water for 24 hours. After removal of the globulins by centrifugation, this final solution was virtually free of any OAA carboxylase activity in the absence of added divalent cations. The Mn^{++}-activated enzyme, which could readily be demonstrated, is apparently quite different from the globulin, but similar to the OAA carboxylases found in the other sources shown in Table II.

The figures given in Table II are merely representative. Various preparations from the same source differ considerably among themselves, 100 per cent variation being not unusual. This is hardly surprising, since no attempt was made to control or even to get information on the previous history of the sources. One apparently inactive preparation was obtained from spinach, potato, and cauliflower respectively. In each of these cases three out of four preparations were active. The comparative values of the activity from various sources have a rough validity, as far as indicating the efficacy with which OAA carboxylase can be obtained in demonstrable form. It should be indicated, however, that the ammonium sulfate precipitation procedure employed was chosen for its convenience and may not represent the best procedure for even the first precipitation of the enzyme. In the case of parsley root and wheat germ this is already known not to be the case.

Of the sources examined, wheat germ, parsley root, and parsnip contained the enzyme most abundantly. Of these, wheat germ appears to be the most convenient source, because of its availability as a dry powder Radishes, carrots, cucumber pulp, and beets are also good sources. Legumes, potatoes, spinach, cabbage, and cauliflower are relatively poor sources. Of the fourteen vegetables examined, which included representatives of seven families, OAA carboxylase was found in every case. It seems highly probable that a large number of other plant OAA carboxylases could be found on further examination, at least among related species. Among the fruits (apples, grapefruit, and bananas) findings have hitherto been uniformly negative, but this may be due to inadequacy of procedure and not to absence of the enzyme.

It seems significant that the enzyme is present in a variety of plant organs (roots, tubers, leaves, and seeds), and its occurrence therefore does not seem limited to any particular structure. This wide distribution of OAA carboxylase in different plant species and organs may well be indicative of the wide-spread occurrence of the Wood-Werkman reaction in the tissues of higher plants.

A number of preliminary tests were run on the preparations described in this paper, to detect enzymes possibly related to OAA carboxylase. To date, it has been found that oxalosuccinic carboxylase is present in wheat germ, beets, and peas as well as in parsley root, where it was previously demonstrated. The occurrence of a malic dehydrogenase active with TPN has been reported for parsley root (3), and further observations on the distribution of this enzyme will appear in another communication.

SUMMARY

A procedure has been described for detecting the presence of oxalacetic carboxylase in plant sources. The enzyme has been found in thirteen different species of higher plants. It has been obtained from roots, tubers, leaves, and seeds. On the basis of these findings it is concluded that OAA carboxylase has a wide distribution in the tissue of higher plants.

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THE RELATION TO AGE AND FUNCTION OF REGIONAL VARIATIONS IN NITROGEN AND ASH CONTENT OF BOVINE BONES

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The present study was undertaken to determine some of the causes of variations noted in nitrogen content of bone specimens from humans of various ages who presumably had no disease of the skeletal system. The inorganic constituents of bone have been extensively investigated, but, with very few exceptions (1–4), since the early years of this century there have been no references in the literature to the nitrogen content of bone. It was of interest to us to determine whether analysis of a single specimen of bone would yield information regarding the skeletal system in general or whether each bone might have a characteristic ratio of ash to nitrogen.

Source of Materials—Preliminary work indicated that large bones were necessary to supply samples of the needed size. Beef bones were therefore selected for this work and were obtained from local abattoirs.

Preparation of Samples—Fresh bones were thoroughly cleaned with a knife by scraping off all muscle, fat, periosteum, and fibrous and other adherent tissues, and stored in a refrigerator. Before removing portions for analysis, the surface of the bone was further cleaned by scraping, until it presented a uniform and polished surface. All cancellous bone and endosteal tissue were chiseled off the inner surface of each sample. With a surgical power saw of the Albee type, small cubes of bone weighing about 100 mg. and approximately 3 mm. on an edge were cut from the full thickness of the cortex. In areas of thin cortex, dimensions were altered to provide samples of about 100 mg. in weight, which were found suitable for either ash or nitrogen determinations of requisite accuracy. Care was taken to avoid burning the edges of the samples while cutting, but when this did occur, all burned edges were removed with a dental burr. The cubes were partially dried over calcium chloride in an evacuated desiccator for 24 hours at room temperature. This preliminary drying was found necessary; otherwise a gelatinous surface formed on the bone cubes when they were placed in the 105° oven. After this preliminary drying, dehydration was completed at 105° for 3 days (72 hours) at atmospheric pressure; the dried samples were stored *in vacuo* over calcium chloride. This drying procedure was very satisfactory for the size of the samples chosen; at 105° for 10 days, only 0.5 per cent more weight loss occurred. Samples weighing more than 1 gm. were not adequately dried by this procedure. Bone thus dried is very hygroscopic.

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Chemical Analyses—Samples were ashed by heating 12 hours in a muffle furnace at 600°. Longer heating at this temperature caused no further loss in weight. After cooling to room temperature in a desiccator, the ash was determined gravimetrically. Nitrogen content of bone was determined by the gasometric micro-Kjeldahl procedure of Van Slyke (5).

Procedures

The long bones were sampled in various ways until experience indicated the most satisfactory method. In what we have termed "block sampling," areas in the proximal, middle, and distal portions were blocked out in sufficient size to provide in each block twenty or more samples for nitrogen analysis and an equal number for ash analysis. For "ring sampling," a ring of bone 3 mm. thick was cut at the desired point and then subdivided into equal segments about the circumference, so that all the samples were of adequate size for analysis. Adjacent rings were used for nitrogen and ash determinations. It soon became apparent that the most satisfactory method was that which we term "longitudinal strip sampling," in which the bone shaft was cut into longitudinal strips and then subdivided into suitable samples. Adjacent strips were used for nitrogen and ash analyses. "Longitudinal spot sampling" consisted of selecting from a longitudinal strip a few areas in which, on the basis of previous experience, we should expect to find representative changes in composition of the strip as a whole. "Depth sampling" consisted of splitting cortical bone at its mid-point into endosteal and periosteal samples. Bony tuberosities were examined by combined depth and ring sampling. When strip sampling methods were used, the region of bone from which the strip was obtained was carefully noted and appropriately described as anterior, posterior, medial, or lateral. The position of the sample in relation to the two ends of the diaphysis was recorded. When the ring sampling method was used, the longitudinal position of the ring was noted as well as the circumferential location of the sample used.

Variation inherent in the error of the methods used, in contrast to variation due to differences in composition of the specimens analyzed, was estimated by performing repeated duplicate analyses of homogeneous bone powder obtained by sawing the bone with a hacksaw and mixing the sawdust thoroughly. The powder so obtained consisted largely of particles between 80 and 100 mesh, with some finer than 100 but none larger than 80 mesh.

Steer radii were studied by block, ring, longitudinal, depth, and spot sampling procedures. The ulna, humerus, and tibia were studied by longitudinal and spot sampling methods. Because of local areas of high nitrogen values that at first seemed irregular in occurrence, the regions of insertion of muscles and tendons were investigated by ring and depth sampling. The tuberosities of the humerus were used to exemplify this condition. The bones studied were obtained from a 4 week-old calf, one 18 month- and one 2 year-old steer, a 3 year-old cow, and a 5 year-old bull.

Spot sampling analyses were made on rib and scapula as representative flat bones, and the results were compared with those from long bones.

Results

Analyses of cortical bone from the proximal, mid-, and distal region of a steer radius by block sampling, in which twenty to forty adjacent samples constituted a block, revealed for nitrogen content average values of 4.14, 4.04, and 4.52 gm. per cent with coefficients of variability of 2.8, 2.8, and 2.7 per cent respectively. The ash contents in the same order were 68.9, 70.5, and 66.6 gm. per cent with coefficients of variability of 2.2, 1.9, and 2.3 per cent respectively. Similar duplicate analyses of ten samples of mid-region bone powder, well mixed to insure homogeneity, showed an average nitrogen content of 3.95 gm. per cent with a coefficient of variability of 0.65 per cent, and an average ash content of 70.9 per cent with a coefficient of variability of 0.36 per cent. The differences between the averages for both nitrogen and ash content of the blocks from the mid-region and the distal region were more than 3 times the standard deviation of each, suggesting that this difference was significant. Subsequent studies in which consecutive longitudinal samples were analyzed demonstrated that this difference in variability of samples between bone powder and bone blocks was due, not to random differences in samples obtained from the blocks, but to an orderly change in nitrogen and ash content correlated with longitudinal position, as shown in Table I and Fig. 1.

Depth sampling of cortical bone from a region free of tuberosities showed a uniformity of composition in depth. For, when blocks comprising twenty samples each were further divided into endosteal and periosteal bone samples and analyzed, no significant differences were found between these two regions within a single block. The average nitrogen content was 4.06 gm. per cent for periosteal and 4.00 gm. per cent for endosteal bone. For nitrogen the coefficient of variability was 2.2 per cent for periosteal and 3.2 per cent for endosteal bone. For ash, the average content for periosteal bone was 69.7 gm. per cent and for endosteal bone 71.4 gm. per cent, with respective coefficients of variability of 1.7 and 1.1 per cent. This uniformity of composition in depth of cortical bone

TABLE I

Representative Data from Various Long Bones, Showing Reciprocal Relation between Nitrogen and Ash Content

Each diaphysis analyzed showed a region of minimum nitrogen and maximum ash, with a gradual increase in nitrogen content as either end of the shaft is approached from this region. The relative position of each sample analyzed in the longitudinal strips used is shown approximately by placing the analytical data opposite the appropriate arbitrary position number. All analyses were made on thoroughly dried samples. The values are given in gm. per 100 gm.

Position No.	Radii										Tibiae						Humerus				Ulna	
	Posterior		Anterior								Anterior						Posterior		Lateral		Medial	
	Steer 1, 2 yrs.		Calf, 1⅓ mos.		Steer 2, 1⅓ yrs.		Cow, 3 yrs.		Bull, 5 yrs.		Calf, 1⅓ mos.		Cow, 3 yrs.		Bull, 5 yrs.		Steer 1, 2 yrs.		Steer 1, 2 yrs.		Steer 1, 2 yrs.	
	N	Ash	N	Ash	N	Ash	N	Ash	N	Ash	N	Ash	N	Ash	N	Ash	N	Ash	N	Ash	N	Ash
1	5.05	66.2																				
2	5.01	66.6	5.32	63.8	4.74	64.2	4.64	67.7	4.43	70.3	5.10	57.3	4.45	64.7	4.38	70.1					4.67	66.1
3	4.51	67.6															4.19	70.1				
4	4.31	68.2																	4.39	68.2		
5	4.15	69.2	4.61	68.8	4.34	70.8							4.32	70.2							4.77	67.4
6	4.14	69.8					3.88	71.5	3.54	74.3	4.74	66.1										
7	4.14	69.7			3.75	72.0																
8	4.15	69.7	4.24	70.1									4.14	70.8	4.13	71.5	3.77	72.3	4.04	70.7	4.38	68.6
9	4.18	70.0			3.70	71.8	3.74	72.2														
10	3.99	70.0							3.50	74.4	4.32	69.4									4.42	67.8
11	4.14	70.4	4.26	70.3	3.78	72.4							4.08	70.7								
12	4.17	70.0					3.95	71.6													4.28	69.4
13	4.25	70.3	4.42	68.7	3.90	72.3			3.76	72.7			4.02	71.2			3.78	73.1	3.58	72.9		
14	4.24	70.1									4.38	69.2									4.10	69.5
15	4.24	70.4	4.68	68.4	3.99	71.9	4.24	70.0					4.05	71.8	3.95	71.6						
16	4.31	70.2			4.19	71.1			4.18	71.6											4.11	70.7
17	4.64	68.9			4.05	70.8							3.99	72.6	4.03	71.9						
18	4.66	67.4	4.63	67.8	4.27	70.5	4.43	69.0			4.28	68.7					3.80	72.6	3.54	73.7	4.51	69.5

19	4.27	67.3			4.51	69.8			4.14	71.0												
20	4.54	66.4			4.63	68.7							4.15	72.2	4.07	72.5						
21	4.49	68.0	4.71	65.6	4.77	68.4	4.39	68.8													4.76	66.9
22	4.60	67.6			4.75	67.3			4.32	69.7												
23	4.63	67.7			4.86	66.5					4.62	67.8	4.23	70.7	4.02	72.0	3.84	72.1	3.72	72.5		
24	4.65	67.2	4.92	66.2	4.81	65.7	4.40	68.3													4.99	65.8
25	4.68	67.0			4.91	64.7			4.46	69.3												
26	4.68	66.8	5.09	64.4									4.39	69.1								
27	4.74	66.6			4.97	62.7	4.53	67.0									4.81	67.5			5.02	63.7
28	4.72	66.3	5.17	61.6															4.08	69.5		
29	4.70	66.0									5.23	60.6	4.60	66.2	4.72	68.3						
30	4.84	65.4	5.37	56.9	5.25	58.1	4.86	64.5	4.76	66.9											5.47	57.9
31	4.96	65.1																				

was found in all other bone samples analyzed in this fashion when the sample did not include a tuberosity or point of insertion of a muscle.

In Table I are shown representative results of longitudinal sampling studies of the anterior and posterior aspects of a steer radius, and the anterior aspect of calf, cow, and bull radii. Analyses in similar fashion are shown of the anterior aspect of a steer ulna, the lateral and posterior aspects of á steer humerus, and the anterior aspect of the tibiae of a calf, a cow, and a bull. The pattern of variation of nitrogen and ash content of the various diaphyses will be noted to be alike for all the bones.

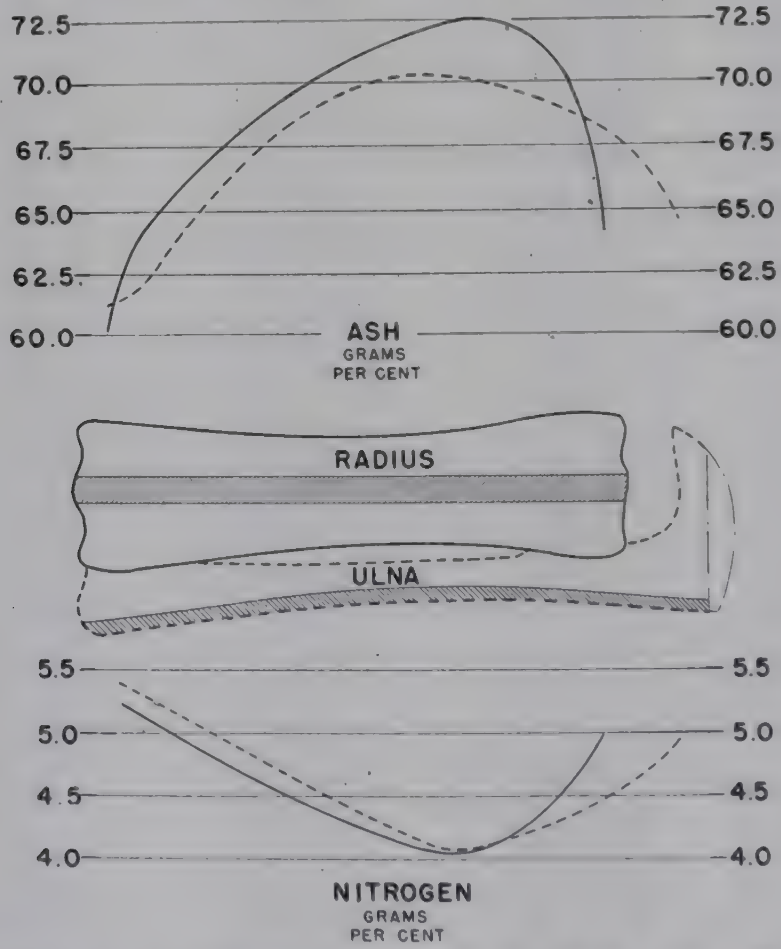

Fig. 1. Analyses for nitrogen and ash of the radius and ulna. The locations of the strips analyzed are shown by the shaded area in each bone. The data for the radius are represented by the solid line, those for the ulna by the broken line.

Fig. 1 demonstrates the varying ash and nitrogen content of the radius and ulna in relation to each other *as the bones exist in the animal.* It will be noted that the region of lowest nitrogen content exists in the same anatomical cross-section of the extremity but at different longitudinal proportions of each bone. This relation was observed in each of four pairs of radii and ulnae studied. Similar longitudinal variations in nitrogen and ash content were found in all long bones studied.

In Fig. 2 are shown the nitrogen and ash percentage of various regions in the humerus through a tuberosity; again a consistent trend in the results is noted. The results of analyses of the scapula are shown in Fig. 3, and of the rib in Fig. 4.

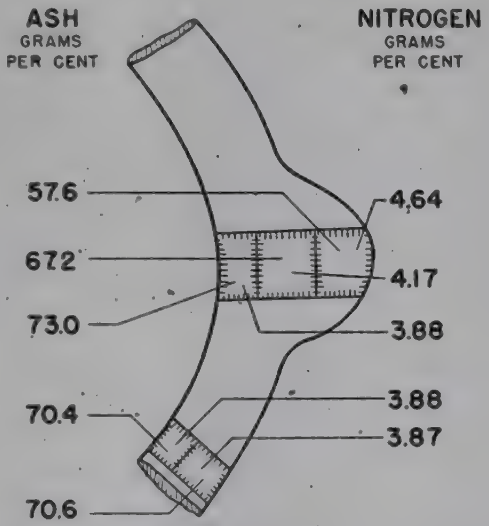
ASH
GRAMS
PER CENT
NITROGEN
GRAMS
PER CENT
57.6
67.2
73.0
70.4
70.6
4.64
4.17
3.88
3.88
3.87

FIG. 2. Analyses for nitrogen and ash through a tuberosity of the humerus, showing the increased nitrogen content of the tuberosity as compared with cortical bone in near-by areas. 18 month-old steer.

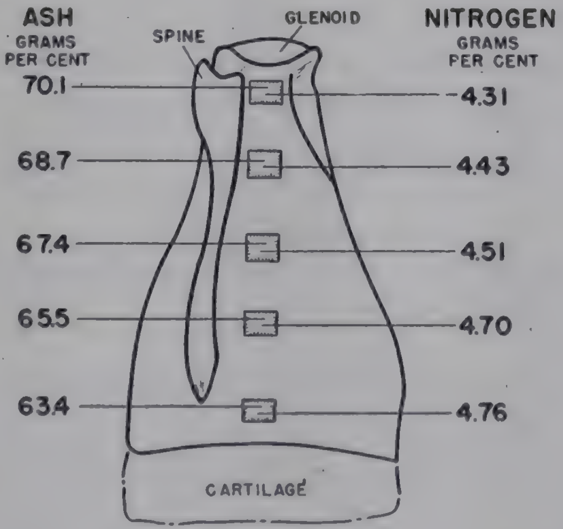
ASH
GRAMS
PER CENT
SPINE
GLENOID
NITROGEN
GRAMS
PER CENT
70.1
68.7
67.4
65.5
63.4
4.31
4.43
4.51
4.70
4.76
CARTILAGE

FIG. 3. Schematic representation of scapula of cow, showing variations in nitrogen and ash content in different regions of the bone.

It will be noted that the data in Table I and Fig. 1 show variations in both nitrogen and ash content when one region of a bone is compared with another. A significant difference exists in nitrogen content between a region approaching the epiphysis in a young bone and the region containing the minimum nitrogen content. Further, as one progresses from this region of minimum nitrogen content toward either end of the diaphysis, the nitrogen content increases. The same phenomenon is noted in relation to the ash content, except that a maximum ash content is found in the region of minimum nitrogen and the percentage of ash decreases as one progresses from this point toward either epiphysis. It will be further noted that for both ash and nitrogen content the rate of change per cm. of diaphyseal shaft varies not only for different bones but also for the same bone when growth at the two ends is unequal, as best exemplified by the radius. The pattern of change appears to be consistent and relatively constant for each bone, and bones of similar growth characteristics have similar analytical patterns. The data also indicate that these changes in composition extend through the thickness of cortical bone, as shown by the identical analytical values for endosteal and periosteal bone. That all bone is not of constant composition in any given region is shown by the analyses through the tuberosity of the humerus given in Fig. 2. Ring sampling of bone shafts and comparison of strips from longitudinal and lateral regions show that, although the circumferential composition is not constant at any given ring, the variation longitudinally is of a constant pattern. These data together show that bone is unexpectedly complex in its structure and an attempt is made to elucidate some of these points in the discussion.

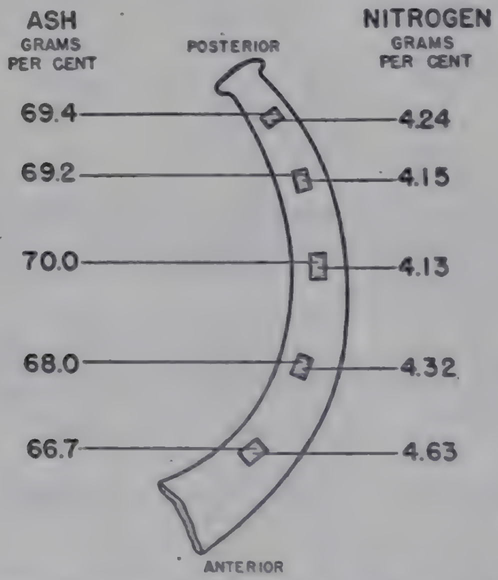

Fig. 4. Schematic representation of rib of cow, showing variations in nitrogen and ash content in different regions of the bone.

DISCUSSION

Examination of the analytical results suggests that composition of bone bears some reasonably direct relation to the age of the animal. For in the 4 week-old calf the nitrogen content of a longitudinal strip of the radius was 4.76 gm. per cent, that of the 2 year-old steer 4.38 gm. per cent, that of the 3 year-old cow 4.31 gm. per cent, and that of the 5 year-old bull 4.12 gm. per cent. The same sequence of decreasing nitrogen content with increasing age of the animal was observed in those regions of the radii which contained a minimum of nitrogen. Thus the region of minimum nitrogen content in the young calf was 4.24 gm. per cent, in the 2 year-old steer 3.70 gm. per cent, in the 3 year-old cow 3.74 gm. per cent, and in the 5 year-old bull 3.50 gm. per cent. The values for ash content varied inversely with the nitrogen content and also exhibited a similar correlation with age of the animal. It is believed for reasons set forth later that comparison of these minimum points is more valid than comparisons of the entire strips, particularly when the bone contained open epiphyses. In addition to this general relation between the age of animal and bone composition, there can be suggested a reasonable basis for variation within single bones which also appears to correlate with the age of the region under consideration.

The long bones such as the radius, humerus, and femur begin ossifying from a single center at about the mid-point of the fetal bone. Ossification later proceeds also from centers at each epiphysis, which process continues during the longitudinal growth of the bone. The rib begins to ossify from a center which comes to lie at about the angle of the full grown rib. It is of interest in this light to examine the region in which the lowest content of nitrogen has been found. For the ribs studied the region of minimum nitrogen content was found at about the angle of the rib, an excellent correspondence with the region of fetal ossification. Further correspondence is found by comparing the ulna and its adjacent radius. In the fetus the original centers of ossification of the two bones appear to lie in the same anatomical cross-section which is in approximately the mid-region of each fetal bone. Approximately two-thirds of the increase in length of the radius occurs at the distal end and one-third at the proximal end; for the ulna the ratio of growth of the distal end to the proximal end is approximately 3:2. The epiphyseal centers of ossification are first noted distally and some time later proximally, but final union occurs in the reverse order, the proximal centers fusing first and the distal centers later. Thus in the young adult radius the region containing the original fetal center would lie at a distance about one-third the length of the diaphysis from the proximal end and in the ulna about four-tenths the length of the diaphysis from the proximal end. With both epiphyses closed, the bone about the proximal epiphysis would be somewhat older than that about the distal epiphysis. The analytical data given show the point of minimum nitrogen content of the radius and ulna to lie in the same anatomical cross-section, and in each bone at about the region which would correspond to the region of the fetal center of ossification. Further the nitrogen content about the proximal epiphysis of each bone is less than at the distal epiphysis. Again there is seen a corresponding increase with age.

Longitudinal growth in a distal direction is the same for ulna and radius. It will be noted that both the rate of change of nitrogen content per cm. of diaphyseal shaft and the percentage of nitrogen content are almost the same for both bones from their distal ends to the regions of minimum nitrogen content. If one progresses proximally from this point, a marked difference is noted in the rate of change of nitrogen content for the two bones, but if one selects any given point proportionately equidistant from the minimum nitrogen region, again the values for nitrogen are found to be about the same, although if the mid-point of the respective portion is selected, about 20 per cent of the total length of the shaft of the ulna has been traversed and but 13 per cent of the length of radius. Whether growth proceeds more rapidly or more slowly does not seem to affect significantly the nitrogen content. It would thus appear that if the age of the bone observed and the geometric position within the bone from which the analytical sample was obtained are taken into account, a valid comparison may be made between bones of different animals, and standards set up for comparison need range over a much smaller interval than previously reported analyses of nitrogen content of bones would indicate.

The variation in nitrogen and ash of cortical bone along the shafts of long bones we have also found in analyses of human bones. In humans also a decreasing nitrogen content was found with increasing age and in the eldest subject studied variation in nitrogen content was found to be at a minimum. In every instance the human bone studied had a greater nitrogen content than did the beef bones, but the pattern of variability was similar in the two species.

If the results of analyses of cortical bone about open epiphyses are examined, it will be observed that the nitrogen content is fairly constant at approximately 5.3 gm. per cent. This suggests that new bone has a reasonably constant nitrogen content. The values given in our data must be considered only as an approximation, because there are very considerable technical difficulties encountered in obtaining suitable specimens of cortical bone near the epiphysis. Variations in ash content about the epiphyses were greater than those observed for nitrogen content. On the basis of data available one cannot say whether this is merely a reflection of technical difficulties in sampling or whether a greater latitude actually exists in mineral content. Further work will have to be done to settle this point.

The change in composition in depth which was observed to occur at tu berosities can be explained in at least two ways. It may be that the decreasing ash content of tuberosities reflects a change in composition with a change in function, for at this point the bone is not acting as a rigid supporting mechanism but as a stanchion for the attachment of a tendon. The fact that flat bones, in which structural support is minimum but muscle anchorage is paramount, have a uniformly slightly higher nitrogen content than cortical bone of long bones would support this explanation. On the other hand the greater tuberosity arises from a separate center of ossification and the higher nitrogen values encountered here may be merely a reflection of its lesser age. Additional data should shed considerable light on this question.

SUMMARY

1. Analyses for total nitrogen and ash content were made on the cortical bone of bovine radii, humeri, ulnae, tibiae, scapulae, and ribs.
2. Nitrogen content was found to be at a maximum at either end of the diaphyses and at a minimum at an intermediate point characteristic for each bone studied. Ash content varied likewise, but in a reciprocal fashion.
3. The rate of increase in nitrogen content from the area of minimum nitrogen content to either end was regular, and characteristic for each long bone studied.
4. Nitrogen content of cortical bone decreased with increasing age of the animal, while ash content increased. With increasing age of animals the composition of bone became more uniform.
5. Regional variations in nitrogen and ash content within a single bone could be correlated with differences in age of the different regions.
6. The nitrogen content of bone about open epiphyses appeared to approach a limiting value. Evidence for a similar limiting value for ash content was not clear.
7. Tuberosities of long bones contain more nitrogen and less ash than the endosteal bone at this area and less than cortical bone in adjacent areas, where no difference was found in composition between periosteal and endosteal bone.
8. Flat bones show a similar variation in composition.
9. The significance of the findings is discussed.

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ESTERIFICATION OF INORGANIC PHOSPHATE COUPLED TO ELECTRON TRANSPORT BETWEEN DIHYDRODIPHOSPHOPYRIDINE NUCLEOTIDE AND OXYGEN. I*

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Measurement of the efficiency of aerobic phosphorylation by Ochoa has disclosed that at least 2 and possibly 3 molecules of inorganic phosphate become esterified during the passage of a pair of electrons from substrate to oxygen during the complete oxidation of pyruvate in cat heart extracts (2). Thermodynamic analysis of these data (2, 3) predicts that one or more of these phosphorylations may occur during the passage of electrons from the first electron acceptor (pyridine or flavin nucleotides, etc.) to oxygen. Up to the present, a direct demonstration of phosphate esterification during the course of electron transport between a primary electron acceptor and oxygen has not been reported.

It would appear that the most obvious approach to such a demonstration would lie in the study of electron transport between the pyridine nucleotides and oxygen in tissue preparations known to be capable of esterifying inorganic phosphate during oxidations over the Krebs tricarboxylic acid cycle. There are two pyridine nucleotide-linked oxidative steps in the Krebs cycle; namely, the oxidation of isocitrate to oxalosuccinate and the oxidation of malate to oxalacetate. The other oxidative steps in the cycle are less suited for this approach, since the primary electron acceptors in these oxidations are not known.

This problem has been approached by Ochoa (2, 4). Unpublished experiments quoted by Ochoa (2), in which large amounts of dihydrodiphosphopyridine nucleotide ($DPNH_2$) were incubated aerobically with cat heart extracts supplemented with Mg^{++}, adenine nucleotide, inorganic phosphate, fluoride, and glucose as phosphate acceptor, showed large oxygen uptakes but no net esterification of phosphate. In addition, Ochoa found that the oxidation of isocitrate to oxalosuccinate (a triphosphopyridine-linked oxidation) also failed to cause esterification of inorganic phos-

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phate, although large oxygen uptakes were observed (4). In the latter experiment the further oxidation of α-ketoglutarate was prevented by the use of arsenite as an inhibitor.

These failures to observe coupled esterification, if the experimental conditions were valid, would force the conclusion that the pyridine nucleotide-catalyzed oxidations of the Krebs cycle cause no esterification of phosphate and that the remaining three oxidative steps of the cycle, namely

$$\text{Pyruvate} + \text{oxalacetate} + [O] \rightarrow \text{tricarboxylic acid} + CO_2 \tag{1}$$
$$\alpha\text{-Ketoglutarate} + [O] \rightarrow \text{succinate} + CO_2 \tag{2}$$
$$\text{Succinate} + [O] \rightarrow \text{fumarate} \tag{3}$$

must account for all the esterification of phosphate observed during complete oxidation of pyruvate. As a corollary of this conclusion each of these three oxidations must then be accompanied by the esterification of as many as 4 or 5 molecules of inorganic phosphate, a yield which approaches complete thermodynamic efficiency of the conversion of energy yielded in these oxidations into phosphate bond energy.

It appeared to us that the negative results reported by Ochoa in his study of phosphate esterification coupled to the pyridine-catalyzed oxidations might possibly have been a reflection of some conditions of his experiments which were adverse to the demonstration of phosphorylation by measurement of net phosphate uptake. In this paper are described experiments in which P^{32} was used as a tracer to establish that esterification of inorganic phosphate is coupled to electron transport between $DPNH_2$ and oxygen in a system known to cause esterification of inorganic phosphate coupled to Krebs cycle oxidations.

In Paper II (5) these findings are confirmed by a somewhat different approach and the properties of the coupling system more thoroughly examined.

EXPERIMENTAL

Preparative—The diphosphopyridine nucleotide (DPN) preparation used as starting material (Schwarz Laboratories) was 47 per cent pure according to spectrophotometric measurement (6, 7). It was first freed of mononucleotide impurities by a method used by Warburg, Christian, and Griese (8) in the purification of triphosphopyridine nucleotide (TPN). This involved making a saturated solution of the DPN in cold 0.1 N methanolic HCl and precipitating the DPN by the addition of ethyl acetate. The DPN recovered (purity of 55 to 60 per cent) was then reduced with sodium hydrosulfite and the sodium salt of $DPNH_2$ isolated according to the method of Ohlmeyer (9) and dried *in vacuo*. The purity of the dry compound was found to be 71.6 per cent on the basis of the composition and molecular extinction coefficient given by Ohlmeyer, 6.28 × 10^6 sq. cm. × mole^{-1}. Other preparations of lesser purity were used in preliminary experiments. None of these preparations contained hydrosulfite or other substances capable of reacting with methylene blue.

The sodium salt of DPN of 56 per cent purity, dry weight basis, was prepared by the addition of alcohol to a neutral solution of DPN; *i.e.*, the same method that was used for the isolation of the sodium salt of $DPNH_2$ (sodium hydrosulfite was omitted). Although the yield was poor, this method was adhered to in order to obtain a sample of DPN having approximately the same history of purification as had $DPNH_2$ prepared by Ohlmeyer's method.

The enzyme preparation used consisted of a dilute water suspension of the washed particulate matter of rat liver. Chilled rat liver, 2.7 gm., was homogenized in the cold with 8.0 ml. of cold 0.13 M KCl-0.013 M sodium phosphate, pH 7.8. The homogenate was strained and diluted with an equal volume of the cold buffered KCl. The particulate elements were separated by centrifugation and washed as described by Lehninger and Kennedy (10). The washed residue was then suspended in ice-cold water, made to a volume of 15 ml., and strained. The preparation was stable at 0° for a period of at least 3 hours. This preparation is capable of oxidizing fatty acids and intermediates of the Krebs cycle (10).

Techniques—In all cases except in the anaerobic experiments, the cold enzyme preparation was added last, directly to the otherwise complete reaction mixture in the main compartment of the Warburg vessels, which were kept chilled in ice prior to the addition of enzyme. The sodium salts of $DPNH_2$ and DPN were dissolved in 0.1 M glycylglycine buffer, pH 7.4 (about 5 mg. per 0.1 ml. of buffer), and introduced into the main compartment of the Warburg vessels shortly before the addition of the enzyme.

In the anaerobic experiments, ice-cold enzyme (0.3 ml.) was placed in the side arm of a vessel equipped with a gassing vent. All of the other components of the reaction mixture including the pyridine nucleotide were in the main compartment. The vessel, placed in a chopped ice bath, was then given a preliminary flushing with a stream of nitrogen freed of traces of oxygen by passage through alkaline pyrogallol in an absorption tower. After 2 to 3 minutes flushing, the vessel was temporarily stoppered. An oxygen-carbon dioxide absorbent, containing sodium hyposulfite and sodium anthraquinone-β-sulfonate in alkali (11), was pipetted into the center well which contained a filter paper roll. The vessel was then quickly attached to the manometer and immersed in an ice water bath consisting of a bucket which had been placed in the emptied Warburg bath. This precaution was necessary to minimize inactivation of the enzyme during flushing with gas. The vessel was flushed with nitrogen for 5 minutes at

0°. The ice bath was then replaced with a bath at 30°. The enzyme was then tipped in from the side arm and the reaction mixture incubated for 7 minutes.

The reactions were stopped by the addition of 2.5 ml. of cold 10 per cent trichloroacetic acid. Aliquots of the filtrates were treated with carrier inorganic phosphate and magnesia mixture to remove inorganic P^{32} by essentially the same procedure which is fully described in Paper II (5). The radioactivity of the esterified P^{32} in the supernatants, now essentially free of inorganic P^{32}, was determined by conventional counting apparatus. Under our counting conditions 1 microcurie of P^{32} = 75,000 counts. Inorganic and esterified P were determined by standard methods.

The P^{32} was obtained through the Atomic Energy Commission as $H_3P^{32}O_4$. It was subjected to hydrolysis in 1 N HCl at 100° for 10 minutes and neutralized before use, since the material appeared to contain small amounts of pyro- or metaphosphate.

Esterification of Inorganic Phosphate Coupled to Krebs Cycle Oxidations—The suspensions of the washed particulate fraction of rat liver used in this investigation catalyze all the reactions of the Krebs tricarboxylic acid cycle and fatty acid oxidation (10). The demonstration of coupled phosphorylation in these preparations by measuring *net* uptake of inorganic phosphate has not been possible, since the necessary transphosphorylases required to cause phosphorylation of acceptors such as glucose, creatine, etc., by adenosine triphosphate (ATP) have been removed in the washing procedure. However, it is readily possible to demonstrate the maintenance of the esterified phosphate of ATP coupled to oxidation. In the absence of oxidizable substrate, ATP is quickly dephosphorylated by phosphatases; in the presence of active oxidation of added substrates of the Krebs cycle the level of esterified phosphate may be maintained at or somewhat below the starting level. The use of inorganic phosphate labeled with P^{32} provides proof that such maintenance is the dynamic resultant of dephosphorylation and oxidation-coupled rephosphorylation, since after oxidation has taken place a large part of the P^{32} is found in the esterified P fraction.

In Table I are shown data on oxygen uptake, inorganic P, esterified P, and incorporation of inorganic P^{32} into the esterified P fraction during the oxidation for a short period of Krebs cycle intermediates. The zero time experiment demonstrates the efficacy of the methods used to separate inorganic from esterified P^{32}. It can be seen that the oxidation of all the Krebs cycle intermediates tested caused some degree of maintenance of levels of esterified P compared to the vessel without substrate. The data also show that oxidation of these substrates causes substantial incorporation of inorganic P^{32} into the esterified fraction, ranging from 10 to 39 per cent of the P^{32} originally added as inorganic phosphate. In the absence of added substrate a small amount of P^{32} esterification took place, probably coupled to oxidation of traces of substrate present in the enzyme preparation. It should be stressed that the esterification of phosphate observed on oxidation of any particular intermediate cannot be ascribed entirely to the oxidation of that intermediate to its immediate oxidation product, since all intermediates of the cycle are oxidizable in this system.

TABLE I

Incorporation of Inorganic Phosphate Labeled with P^{32} into Acid-Soluble Esterified Phosphate Coupled to Krebs Cycle Oxidations

The main compartment of the Warburg vessel contained 0.60 ml. of enzyme, 0.10 ml. of $MgSO_4$ (0.005 M),[*] 0.20 ml. of KCl (0.05 M), 0.20 ml. of glycylglycine buffer, pH 7.4 (0.01 M), 0.20 ml. of cytochrome *c* (1.5×10^{-5} M), 0.40 ml. of ATP (0.0012 M), 0.10 ml. of inorganic phosphate labeled with P^{32} (556,200 counts per minute), and 0.20 ml. of substrate (0.01 M). Incubated in air at 30° for 7 minutes after a 4 minute equilibration period.

Substrate	Oxygen uptake	Inorganic P	Esterified P	Incorporation of P^{32} into esterified P
	c.mm.	γ	γ	*per cent*
0 time		100	302	0.088
None	3	240	158	3.25
Malate	15	158	240	24.3
Fumarate		158	236	24.2
Oxalacetate	15	206	196	10.1
cis-Aconitate		182	212	16.3
Citrate		164	232	20.0
α-Ketoglutarate	13	214	196	12.0
Succinate	54	120	284	37.4
Pyruvate		185	211	13.8
" + malate	26	115	287	39.2

[*] The figures in parentheses indicate the final concentration of the component in the complete reaction medium. This notation is used in all the tables.

The values of per cent incorporation of P^{32} given in Table I are only a semiquantitative indication of the rate of esterification of inorganic phosphate. The complexity of the exchanges taking place prevents any simple derivation of rates of formation of newly esterified phosphate, as is clear from the data shown in Table II. In this experiment the oxidation of succinate was allowed to take place under the same conditions as in the experiments of Table I, the reaction being stopped at different time intervals for measurement of oxygen uptake, inorganic phosphate, Δ7 phosphate (easily hydrolyzable groups of ATP), and per cent incorporation of P^{32}. It can be seen that the level of Δ7 P remained essentially constant over the 40 minute period studied, as did the level of inorganic phosphate. However, within 2 minutes 16.1 per cent of the inorganic P^{32} was esterified and at 10 minutes a plateau was reached at which about 55 to 60 per cent of the P^{32} is in esterified form. At the plateau the ratio of esterified to total P^{32} is constant and approximately equal to the ratio of Δ7 P to the sum of Δ7 P and inorganic P. This fact indicates that the P^{32} is completely equilibrated between the pool of inorganic phosphate and the easily hydrolyzable groups of ATP. Hydrolysis of the esterified P fraction for 10 minutes at 100° in 1 N H_2SO_4 converted 89 per cent of the P^{32} into a form precipitable by magnesia mixture.

TABLE II

Time Course of Incorporation of Inorganic P^{32} during Succinate Oxidation

The main compartment of the Warburg vessels contained 0.10 ml. of $MgCl_2$ (0.005 M), 0.10 ml. of KCl (0.05 M), 0.10 ml. of glycylglycine buffer, pH 7.8 (0.02 M), 0.40 ml. of ATP, 0.10 ml. of cytochrome *c* (1.5×10^{-4} M), 0.20 ml. of succinate (0.01 M), 0.10 ml. of inorganic phosphate labeled with P^{32} (141,500 counts per minute). Side arm, 0.50 ml. of enzyme tipped in after 5 minutes equilibration. Total volume, 2.0 ml. Stopped with trichloroacetic acid at the times indicated. Temperature, 30°.

Time	O_2 uptake	Inorganic P	Δ 7 P	P^{32} esterified	$\frac{\Delta 7\ P \times 100}{\Delta 7\ P + \text{inorganic } P}$
min.	*c.mm.*	γ	γ	*per cent**	
0		124	126	0.01	50.4
2	5	116	140	16.1	54.7
5	16	106	142	41.0	57.2
10	32	116	138	53.0	54.3
16	49	112	140	56.8	55.5
22	67	102	140	57.7	57.9
30	97	112	142	61.0	55.9
40	125	116	142	60.6	55.1

* In this table the data on the per cent incorporation of P^{32} were corrected by the factor 1.33 to allow for esterified phosphate lost by coprecipitation during precipitation of inorganic phosphate with Mg^{++}. This approximation suffices for the comparison shown above.

The per cent incorporation of P^{32} in any experiment will depend on the time the reaction is stopped and the ratio of inorganic P to Δ7 P and, of course, on the ability of the oxidation to cause esterification. The per cent incorporations of P^{32} given in this paper and Paper II (5) therefore are only qualitative measures of inorganic phosphate esterification and in general no attempt was made to follow rates of incorporation in the very early stages of the oxidation. Calculation of rates of turnover obtained from incorporation rates in the first stage of the oxidation are obviously complicated by the continued fall in the specific activity of the inorganic P pool due to dilution by inactive P arising from the continuous dephosphorylation of the ATP. For this reason most of the reactions studied in these papers were stopped after the plateau had been attained in control vessels. In these experiments mainly "all or none" effects were examined and the measurement of per cent P^{32} incorporated provided a convenient indicator for such gross differences. The approach in its present form does not lend itself readily to measurement of P:O ratios.

The data presented indicate that oxidation of Krebs cycle intermediates by these enzyme preparations causes esterification of inorganic P^{32} in substantial amount. The enzyme preparations and techniques used were therefore regarded as suitable for the qualitative detection of phosphate esterification coupled to $DPNH_2$ oxidation.

TABLE III

Incorporation of Inorganic P^{32} into Acid-Soluble Esterified Phosphate Coupled to Oxidation of $DPNH_2$

The main compartment of the Warburg vessel contained 0.30 ml. of enzyme preparation and final concentrations of components other than substrates as detailed in Table I in a total volume of 1.0 ml. The sodium salt of $DPNH_2$ of 56 per cent purity was added in 0.1 ml. of glycylglycine buffer as indicated. Incubated in air at approximately 30° for 14 minutes.

Substrate	Concentration	Incorporation of P^{32} into esterified P
	M	*per cent*
None		5.0
Malate	0.01	28.5
$DPNH_2$	0.0005	8.6
"	0.001	13.6
"	0.005	23.5

Phosphorylation Coupled to Electron Transport between $DPNH_2$ and Oxygen—The ease of demonstrating aerobic phosphorylation by experiments such as those described led to preliminary studies with $DPNH_2$ as substrate incubated aerobically with enzyme, Mg^{++}, ATP, cytochrome *c*, and inorganic phosphate labeled with P^{32} under conditions exactly as used above with the Krebs cycle intermediates as substrates. The results obtained with varying concentrations of a preparation of $DPNH_2$ of 56 per cent purity are shown in Table III. It can be seen from the data that incubation of increasing concentrations of $DPNH_2$ caused successively greater incorporations of inorganic phosphate labeled with P^{32} into the esterified fraction above the amount given in the absence of $DPNH_2$. At the highest concentration tested (0.005 M) the degree of incorporation was of the same order of magnitude as that given by the oxidation of malate. In these experiments accurate measurements of oxygen uptake were not possible, since a large part of the oxidation took place during the temperature equilibration period. Although it would have been possible to tip the $DPNH_2$ into the main compartment from a side arm *after* equilibration, it was feared that temperature equilibration of the enzyme in the absence of oxidizable substrate for any length of time might cause extensive inactivation of the enzyme system. These fears were partially justified; in Paper II (5) it is shown that the enzymes responsible for the esterification reaction are quickly inactivated at these temperatures. For this reason oxygen uptake was followed only qualitatively. In the experiments outlined in Table III a rapid oxygen uptake was observed with 0.005 M $DPNH_2$.

TABLE IV

Incorporation of Inorganic P^{32} into Acid-Soluble Esterified Phosphate Coupled to $DPNH_2$ Oxidation

The main compartment of the Warburg vessels contained 0.30 ml. of enzyme, 0.05 ml. of $MgSO_4$ (0.005 M), 0.01 ml. of KCl (0.05 M), 0.20 ml. of glycylglycine buffer, pH 7.4 (0.02 M), 0.20 ml. of ATP (0.002 M), 0.05 ml. of cytochrome *c* (1.5×10^{-5}), 0.05 ml. of inorganic phosphate labeled with P^{32} (486,000 counts per minute), and pyridine nucleotides in a total volume of 1.0 ml. Incubated in air at 30° for 7 minutes. Other conditions indicated below.

Experiment	$DPNH_2$ 0.005 M		DPN 0.005 M		No pyridine nucleotides	
	Inorganic P	Incorporation of P^{32} into esterified P	Inorganic P	Incorporation of P^{32} into esterified P	Inorganic P	Incorporation of P^{32} into esterified P
	γ	*per cent*	γ	*per cent*	γ	*per cent*
Complete system	68	28.3	132	1.99	132	4.65
ATP omitted		9.25		0.53		
Cytochrome *c* omitted	181	1.10	140	1.51		
Mg^{++} omitted	144	1.35	134	0.30		
Anaerobic	104	2.42				
+ 0.018 M NaF	48	37.0				
+ 0.009 " arsenite	150	1.68				
+ malate (0.01 M) (no DPN)					73	33.8
0 time					50	0.047

With the concentration of $DPNH_2$ necessary for obtaining substantial incorporation of inorganic P^{32} established, the experiment was repeated with $DPNH_2$ of higher purity (71.5 per cent) and a more complete study was made of the requirements of cofactors, anaerobiosis, and inhibitors. The results of an experiment are shown in Table IV. As in the experiments of Table III, the incubation in air of $DPNH_2$ with enzyme, Mg^{++}, KCl, ATP, cytochrome *c*, and inorganic phosphate labeled with P^{32} caused the incorporation of a substantial part of the P^{32} into a form not precipitable with magnesia mixture. Here again the extent of incorporation was similar to that shown after oxidation of malate. When oxidized DPN was substituted for $DPNH_2$ at the same concentration as a control for oxidizable substrates which may have been present as impurities in the $DPNH_2$, no significant esterification of P^{32} took place. The esterification taking place with $DPNH_2$ is probably associated with oxidation of $DPNH_2$ by molecular oxygen, since under anaerobic conditions very little esterification took place. This is also confirmed by the finding that omission of cytochrome *c*, the presence of which is essential to obtain oxygen uptake in these preparations when they are oxidizing Krebs cycle intermediates, also causes esterification of P^{32} to drop to a very low value.

Since the reaction media also contained Mg^{++} and ATP, which are essential for the Krebs cycle oxidations, the effects of omission of these components were also examined. Omission of ATP caused a loss of 60 per cent in amount of incorporation; omission of Mg^{++} reduced esterification to an insignificant level. In other experiments, omission of ATP resulted in even lower degrees of P^{32} incorporation. Neither ATP nor Mg^{++} has been implicated in the past in the process of electron transport from $DPNH_2$ to oxygen and addition of these compounds to such systems has not revealed any stimulatory effects (*cf.* (12)).

Fluoride, which is known to prevent "leakage" of esterified phosphate during aerobic phosphorylation in crude tissue extracts by inhibiting phosphatases, caused an increase in P^{32} esterified, as might be expected. Arsenite completely abolished the esterification.

80 per cent of the P^{32} esterified during the oxidation of $DPNH_2$ in experiments of this type was found to be converted into inorganic phosphate by a 10 minute period of hydrolysis in 1 N HCl at 100°, indicating that the esterified P^{32} is largely located in the easily hydrolyzable groups of ATP.

The data on P^{32} incorporation are paralleled by data on inorganic phosphate levels also given in Table IV. In the absence of active incorporation of P^{32} (as in the anaerobic experiment, or in the absence of cytochrome *c* or Mg^{++}, or in the presence of arsenite) there is a large increase in inorganic phosphate on incubation, corresponding to hydrolysis of ATP. In those experiments showing active esterification of P^{32}, there is a much smaller increase of inorganic phosphate due to the continuous esterification of phosphate accompanying the oxidation of $DPNH_2$ which counterbalances the action of phosphatases.

To summarize, then, these experiments indicate strongly that esterification of inorganic phosphate accompanies the passage of electrons from $DPNH_2$ to oxygen and that such esterification requires the presence of ATP, Mg^{++}, and cytochrome *c*.

Attempts to Localize Site of Esterification—Since electron transport between $DPNH_2$ and oxygen in this system involves cytochrome *c*, as indicated by the requirement of cytochrome, the possibility that the phosphorylation occurred during electron transport between $DPNH_2$ and cytochrome *c* was investigated. A high concentration of cytochrome *c*, 0.001 M, was substituted for oxygen as acceptor, under either anaerobic conditions or aerobic conditions in the presence of 0.001 M cyanide to prevent oxidation by molecular oxygen via cytochrome oxidase. This amount of cytochrome *c* is equivalent to about one-tenth of the $DPNH_2$ added. In order to determine whether this high concentration of cytochrome *c* is inhibitory to phosphorylation, it was also tested aerobically and compared with the lower concentrations previously used. The results are shown in Table V.

TABLE V

Apparent Absence of Phosphorylation during Electron Transport between $DPNH_2$ and Cytochrome c

The Warburg vessels contained 0.30 ml. of enzyme and components as detailed in Table IV in a total volume of 1.0 ml. The sodium salt of $DPNH_2$ (5 mg.) was added in 0.10 ml. of glycylglycine buffer. Incubated in air or anaerobically as indicated at 30° for 7 minutes. Other conditions indicated below.

	Per cent of inorganic P^{32} incorporated into esterified P	
	1.5×10^{-5} M cytochrome *c*	1.0×10^{-3} M cytochrome *c*
Complete system (aerobic)	24.8	15.2
Pyridine nucleotide omitted (aerobic)	4.23	4.22
Complete system + cyanide (0.001 M) (aerobic)	0.52	0.36
Same, anaerobic (no cyanide)	1.91	1.81

Although the presence of such high concentrations of cytochrome *c* caused some inhibition of phosphorylation under aerobic conditions, the results under anaerobic conditions are unmistakably negative. In the anaerobic experiment and in the experiment in which cytochrome oxidase was inhibited by cyanide the cytochrome *c* was largely reduced, as indicated by the gross color change; however, no esterification comparable to that found in the active aerobic system was found. The amount of cytochrome *c* used as acceptor, 1 micromole, could accept electrons equivalent to 11.2 c.mm. of oxygen uptake if completely reduced. The esterification of inorganic P^{32} coupled to this amount of oxygen uptake if Krebs cycle intermediates are used as substrates is ordinarily readily detectable by the methods used, as may be seen in Table II. For this reason the negative findings obtained in these experiments and others designed for the same purpose (5) have been felt to be of some significance.

Attempts have also been made to determine whether esterification of phosphate accompanies passage of electrons from cytochrome *c*, which is involved in the phosphorylative oxidation of $DPNH_2$, to oxygen. In systems containing enzyme, cytochrome *c*, Mg^{++}, ATP, buffer, and inorganic phosphate labeled with P^{32}, the cytochrome *c* was reduced non-enzymatically *in situ* by substances such as ascorbate, glutathione, cysteine, hydroquinone, and *p*-phenylenediamine (13, 14), resulting in large oxygen uptakes. The oxidation of malate served as a control, giving the usual high degree of P^{32} incorporation (Table VI).

TABLE VI

Study of Phosphate Esterification Coupled to Electron Transport between Reduced Cytochrome c and Oxygen

The Warburg vessels contained 0.50 ml. of enzyme, 0.10 ml. of $MgCl_2$ (0.005 M), 0.10 ml. of KCl (0.05 M), 0.10 ml. of glycylglycine buffer (0.02 M), 0.20 ml. of cytochrome *c* (2×10^{-5} M), 0.40 ml. of ATP (0.002 M), inorganic phosphate labeled with P^{32} (361,000 counts per minute), cytochrome *c* reductants at 0.01 M, and malate at 0.01 M as indicated. Total volume, 2.0 ml. Time, 15 minutes after 4 minutes equilibration at 30°.

Substrate	O_2 uptake	P^{32} esterified
	c.mm.	*per cent*
None (0 time)		0.16
"	2	1.12
Malate	30	28.2
Ascorbate	76	7.4
Glutathione	56	1.10
Cysteine	70	1.10
Hydroquinone	71	0.90
p-Phenylenediamine	84	1.80
Malate + ascorbate	94	18.2
" + glutathione	70	14.3
" + hydroquinone	98	1.80
" + *p*-phenylenediamine	81	0.96

Although there was an extensive flow of electrons over the cytochrome system as indicated by the oxygen uptake when cytochrome *c* was reduced by the above reagents, there was no esterification of inorganic phosphate except in the case of ascorbic acid which showed a small incorporation. However, it may not be immediately concluded that this phase of electron transport from $DPNH_2$ to oxygen does not cause esterification of phosphate. As can be seen from the data, hydroquinone and *p*-phenylenediamine when incubated together with malate appeared to abolish the esterification obtained with malate alone, whereas the other reducing agents inhibited to different degrees. It is possible that such inhibition results from a competition between malate and the reducing agents for available cytochrome *c* and it is also possible that hydroquinone and *p*-phenylenediamine or their oxidation products may have "decoupling" effects, as are noted in Paper II (5). The positive esterification noted with ascorbate alone may be of significance; however, the instability of dehydroascorbic acid may give rise to other metabolites capable of phosphorylative oxidation in such a system.

Localization of the site of esterification of inorganic phosphate along the chain of electron carriers between $DPNH_2$ and oxygen has therefore not been achieved by the relatively simple experiments outlined; however, these experiments have served to define some of the problems involved.

DISCUSSION

The data presented in this paper demonstrate that inorganic phosphate is esterified during the aerobic incubation of $DPNH_2$ with an enzyme preparation known to cause coupled esterification of inorganic phosphate during oxidations in the Krebs cycle. This reaction requires the presence of ATP, Mg^{++}, cytochrome *c*, and oxygen. The requirement of the latter two components and the oxygen uptakes which were observed qualitatively allow the tentative conclusion that the esterification is associated with transport of electrons from $DPNH_2$ over the cytochrome system to oxygen.

These qualitative findings have obvious implications not only in the localization of the sites of the aerobic phosphorylations occurring during the oxidation of pyruvate over the Krebs cycle but also raise some questions concerning the nature of the enzymes involved in electron transport between $DPNH_2$ and the cytochrome system.

There are two points of possible uncertainty in the experiments quoted. The first involves the question of the purity of the $DPNH_2$ samples employed. Although control experiments with the oxidized form of the nucleotide, having approximately the same history of purification, showed no significant esterification, it is conceivable that an oxidizable impurity may have been formed by the action of hydrosulfite during the preparation of the $DPNH_2$. A second point on which more information is necessary is the exact measurement of oxygen uptake during such experiments. This becomes of some importance in considering the necessity of ATP and Mg^{++} for the esterification. These factors are not known to be involved in any obligatory way in DPN-linked oxidations over the cytochrome system. If esterification is not an obligatory accompaniment of oxidation, this would be revealed by more exact measurements of oxygen uptake than were possible in this study.

The experiments reported in this paper offer no immediately obviou explanation for Ochoa's failures to detect phosphorylation other than th finding that arsenite, which Ochoa used to prevent further oxidation o α-ketoglutarate in his experiments with the TPN-linked oxidation, is strongly inhibitory to the esterification.

With the fundamental data reported here as a basis, a somewhat different approach to the problem was devised which completely confirmed these findings and which allowed more systematic and convenient study of the phenomenon reported. These data are reported in Paper II (5). As will be seen, the two approaches supplement and confirm each other, providing proof that esterification of phosphate occurs between $DPNH_2$ and oxygen in the enzyme preparations under study.

Experimental localization of sites of esterification in the chain of electron transport enzymes will offer some practical difficulties, as is indicated by the experiments described.

SUMMARY

The aerobic incubation of dihydrodiphosphopyridine nucleotide of 71.5 per cent purity with the washed particulate matter of rat liver in the presence of ATP, Mg^{++}, and cytochrome *c* causes substantial incorporation of inorganic phosphate labeled with P^{32} into the esterified phosphate fraction. 80 per cent of the esterified P^{32} can be liberated as inorganic phosphate by a 10 minute period of hydrolysis in 1 N HCl at 100°, indicating that adenine nucleotide is the phosphate acceptor. Omission of any one of the components enumerated results in failure of esterification, as does anaerobiosis. Fluoride increased the amount of P^{32} esterified; arsenite completely inhibited the response. These facts permit the tentative conclusion that esterification of inorganic phosphate accompanies the transport of electrons from $DPNH_2$ to oxygen under the conditions studied. It was not possible to locate by decisive experiments the sites of esterification in the electron transport chain.

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ESTERIFICATION OF INORGANIC PHOSPHATE COUPLED TO ELECTRON TRANSPORT BETWEEN DIHYDRODIPHOSPHOPYRIDINE NUCLEOTIDE AND OXYGEN. II*

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In Paper I (3) evidence was given to show that inorganic phosphate is esterified when dihydrodiphosphopyridine nucleotide ($DPNH_2$) is incubated aerobically with a particulate fraction of rat liver in the presence of adenosine triphosphate (ATP), Mg^{++}, cytochrome *c*, and inorganic phosphate labeled with P^{32}. The presence of Mg^{++}, cytochrome *c*, ATP, and oxygen was required for the esterification reaction. The data have been interpreted to mean that, as electrons pass from $DPNH_2$ to oxygen over the cytochrome system, inorganic phosphate is esterified. This interpretation is in agreement with the prediction (4, 5) that some esterification of inorganic phosphate may occur during electron transport between primary acceptors and oxygen to account for the known high efficiency of oxidative phosphorylation during pyruvate oxidation (4).

The approach used in Paper I, namely the use of high concentrations of $DPNH_2$ as substrate, does not lend itself to systematic study of the coupled esterification owing to the extremely high concentration of nucleotide required to yield substantial electron turnover, the inability to measure oxygen uptakes accurately, and the inconvenience and expense of preparing large amounts of the reduced nucleotide, which is itself of only limited stability and purity.

The other obvious approach for study of this problem is the use of a diphosphopyridine nucleotide-linked dehydrogenase and its substrate to generate $DPNH_2$ continuously. As the $DPNH_2$ is reoxidized by oxygen, the DPN formed will again be reduced by the substrate and dehydrogenase. However, there is a practical difficulty in this approach. Since it is desired to study only the esterification of inorganic phosphate coupled to electron transport between $DPNH_2$ and oxygen, uncomplicated by oxidative phosphorylation due to oxidation of extraneous substrates, it is necessary to choose a dehydrogenase system, the reaction product of which is not capable of undergoing further oxidation and thereby contributing to phosphorylation events. Although it is possible to inhibit the further oxidation of such

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a product more or less selectively (*i.e.* the oxidation of pyruvate, the product of the lactic dehydrogenase system, can be inhibited by arsenite (6)), it appeared best to avoid the use of inhibitors because of possible additional inhibitory effects on the coupled esterification. For instance, in Paper I (3) it was shown that arsenite completely abolished the esterification coupled to $DPNH_2$ oxidation.

In the course of this work it was found that the β-hydroxybutyrate-β-hydroxybutyric dehydrogenase system, which is DPN-linked (7), was an ideal dehydrogenase system for this study. The dehydrogenase is present in the particulate fraction of rat liver, consisting largely of mitochondria, which contains the phosphorylation system. It shows all the characteristics of a DPN-linked dehydrogenase. The reaction product, acetoacetate, is completely inert in the system and does not undergo further oxidation.

Use of β-hydroxybutyrate as substrate allows continuous generation of $DPNH_2$ in sufficient quantity to allow accurate measurement of oxygen uptake, which can be checked by measurement of acetoacetate formation. There are only traces of endogenous oxidative activity. Using this system we have made a more extensive study of the esterification of inorganic phosphate accompanying electron transport from $DPNH_2$ to oxygen than was possible with $DPNH_2$ as substrate. The data obtained confirm and extend the findings made with the use of $DPNH_2$ as substrate reported in Paper I (3).

Preparative—The sodium salt of *dl*-β-hydroxybutyric acid (BOH) was obtained in crystalline form by saponifying the ethyl ester (Eastman) with an equivalent of 2 N NaOH at room temperature. After completion of saponification the solution was neutralized and brought to dryness *in vacuo* and the crude sodium salt recrystallized from ethanol. The DPN used was obtained from the Schwarz Laboratories. The purity varied from 45 to 60 per cent as tested spectrophotometrically (8). This material appeared not to contain substances grossly inhibitory to any of the reactions examined. Acetoacetate was prepared by the method of Ljunggren (9). The ATP was prepared from rabbit muscle (10). In addition the sodium salt of ATP obtainable from the Rohm and Haas Company was also found suitable.

The enzyme preparations used in this study were essentially those used in the past in this laboratory to study fatty acid oxidation. Two different types of preparations were used. Enzyme I was prepared from rat liver as previously described (3, 11) and Enzyme II was a preparation consisting almost entirely of mitochondria from rat liver isolated by a short modification of the method of Hogeboom, Schneider, and Pallade (12). Chilled rat liver freshly removed from the exsanguinated rat was homogenized in

9 volumes of 0.88 M sucrose, strained, and centrifuged three times at 600 × g, the supernatant being decanted each time from the residue of nuclei, erythrocytes, and intact cells. The supernatant, now cleared of these elements, was made 0.15 M with NaCl by addition of $\frac{1}{9}$ volume of 1.5 M NaCl, allowed to stand 5 minutes in an ice bath, and the agglutinated mitochondria sedimented by 10 minutes centrifugation in a Sorvall angle centrifuge at 2400 × g. The mitochondria were resuspended in 0.15 M NaCl to make a volume equal to that of the first sucrose homogenate and resedimented. They were then suspended in 3 to 4 volumes of 0.15 M NaCl or H_2O as indicated. All the operations were carried out in the cold. If the enzyme preparations are allowed to come to room temperature for even a short period, the phosphorylative activity is lost without affecting the oxidative activity, as will be shown.

The two enzyme preparations described are enzymatically equivalent. Enzyme I contains nuclei, erythrocytes, and some intact cells in addition to mitochondria. The short procedure for isolation of mitochondria described above differs from the published procedure (12) in the addition of NaCl to the 0.88 M sucrose homogenate after clearing it of nuclei, etc., in order to agglutinate the mitochondria, causing them to sediment more rapidly at lower speeds.

Techniques—The reactions described were carried out in standard Warburg vessels, containing alkali in the center well, at 30°. The enzyme was added last to the otherwise complete reaction medium, either by tipping it in from the side arm or by adding it directly as indicated. A temperature equilibration period of 5 minutes was allowed, followed by closing the taps. At the completion of the incubation period, 8.0 ml. of 5 per cent trichloroacetic acid were added to stop the reaction. Aliquots of the filtrates were used for acetoacetate analysis (a modification of the method of Greenberg and Lester (13)), for determination of inorganic and esterified phosphate, and for determination of esterified P^{32}.

The esterified P^{32} was measured by determining the radioactivity remaining in solution after precipitation of inorganic phosphate with magnesia mixture. To 5.0 ml. of the trichloroacetic acid filtrate were added 1.0 ml. of magnesia mixture (0.1 M $MgSO_4$ in 1 M NH_4Cl) and 0.05 ml. 0.1 M phosphate buffer, pH 7.4 (as diluent of the inorganic P^{32}). The mixture was brought to approximately pH 9.0 by addition of 0.20 ml. of concentrated aqueous ammonia (15 N). The contents were stirred in an ice bath until the magnesium ammonium phosphate precipitate began to form. The tubes were left at 0° for at least 4 hours and then centrifuged for 15 minutes. The supernatants were then carefully decanted into tubes containing 0.05 ml. of 0.1 M phosphate and 0.15 ml. of 10 N H_2SO_4. After mixing, 0.20 ml. of 15 N NH_3 was added. The tubes were then stirred in an ice bath again and allowed to stand for at least 4 hours. The tubes were then centrifuged for 15 minutes and the supernatants carefully decanted into clean tubes. Dilutions of these supernatants were then made for the actual counts, which were performed on 1.0 ml. samples with the conventional Geiger-Müller tube and recording apparatus. The separations of inorganic P^{32} from esterified P^{32} made in this way were very effective, leaving behind at the most 0.5 per cent of the inorganic P^{32}. Since most of the esterifications measured involved incorporations of the order of 10 to 80 per cent of the inorganic P^{32} added to the system, the data are not corrected for the small amount of inorganic P^{32} remaining after carrier dilution and two magnesia precipitations. Zero time values are given with some experiments to indicate effectiveness of separation. There are losses of esterified phosphate (ATP) due to coprecipitation with inorganic phosphate during the magnesia treatment. After two precipitations the average recovery of ATP (which has been found not to vary widely) is about 70 per cent of that originally present. The data given are not corrected for these losses. The conditions of magnesia separation described give optimal yields of esterified phosphate. None of the substrates, inhibitors, etc., present in the samples prepared as above appeared to interfere seriously with the separations.

By this technique the determination of esterified P^{32} is quite reproducible between replicate aliquots of a given filtrate and lends itself nicely to routine use.

With the sample and counter geometry used, 1 microcurie of P^{32} was equal to about 75,000 counts. Each Warburg vessel contained between 1 and 6 microcuries of P^{32}.

Requirements for Oxidation of BOH and Coupled Phosphorylation—After a number of preliminary experiments to establish optimal conditions of substrate and cofactor concentrations the requirements for oxidation of BOH to acetoacetate and for the esterification of inorganic phosphate coupled to this oxidation were studied. In Table I is a representative group of data demonstrating that the oxidation of BOH to acetoacetate requires the presence of DPN[1] and cytochrome *c* and that a non-obligatory esterification of inorganic phosphate accompanies the oxidation provided Mg^{++} and

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[1] Sufficient DPN is already present in most enzyme preparations for suboptimal oxidation of BOH, provided the enzyme is added immediately to the main compartment. However, if the enzyme is tipped in from the side arm after a 5 minute temperature equilibration period this DPN is completely destroyed and an absolute requirement of added DPN is then apparent. If the suspension of mitochondria used is very heavy, the requirements of DPN, Mg^{++}, and cytochrome *c* may not be apparent, because sufficient concentrations of these factors will be present. Dilution of such heavy suspensions will result in more or less absolute requirements for the factors mentioned.

ATP are also present. The oxidation of BOH was followed by measuring oxygen uptake and acetoacetate formation. Phosphorylation events were followed by the measurement of inorganic phosphate, easily hydrolyzable phosphate, and the extent of incorporation of inorganic P^{32} into the esterified fraction. As can be seen, there is excellent agreement between oxygen uptake and acetoacetate formation, indicating that no significant oxidation of acetoacetate occurs. Omission of substrate, DPN, or cytochrome *c* caused the oxygen uptake to fall to zero or very low values. These findings are in agreement with known facts concerning electron transport from DPN-linked dehydrogenases to oxygen (*cf.* Potter (14)). However, the additional presence of ATP and Mg^{++}, which are not required for the oxidation and which have no significant effect on the oxidation rate by themselves, caused esterification of inorganic phosphate to occur, as was evidenced by maintenance of the level of Δ7 P and by the extensive incorporation of inorganic P^{32} into the esterified fraction. Esterification of inorganic phosphate did not occur if substrate, DPN, Mg^{++}, ATP, or cytochrome *c* was omitted singly. The data on P^{32} esterification are paralleled by the measurements of the easily hydrolyzable phosphate groups of ATP.

TABLE I

Requirements for Oxidation of β-Hydroxybutyrate and for Coupled Phosphorylation

The main compartment of the Warburg vessels contained 0.10 ml. of $MgSO_4$ (0.005 M),* 0.10 ml. of nicotinamide (0.02 M), 0.10 ml. of glycylglycine buffer, pH 7.5 (0.02 M), 0.10 ml. of *dl*-β-hydroxybutyrate (0.02 M), 0.10 ml. of DPN (0.0005 M), 0.20 ml. of cytochrome *c* (10^{-5} M), 0.40 ml. of ATP (0.0013 M), 0.10 ml. of P^{32} as inorganic phosphate (269,000 counts per minute = 3.6 microcuries), 0.30 ml. of H_2O. Enzyme II, in 0.15 M NaCl (0.50 ml.), added from the side arm after a 5 minute equilibration period, making the total volume of 2.0 ml. When components were omitted, an equal volume of water was substituted. Time, 20 minutes.

	O_2 uptake	Acetoacetate formed	Inorganic P	Δ7 P	P^{32} esterified
	c.mm.	*c.mm.*	γ	γ	*per cent*
Complete system	88	186	180	132	21.6
Same	92	183	172	140	23.0
" 0 time			90	166	0.4
Substrate omitted	0	0	280	30	1.2
DPN omitted	0	0	322	0	0.4
Cytochrome *c* omitted	13	25	269	31	2.1
Mg^{++} omitted	88	190	296	26	0.8
ATP "	94	198	50	12	0.6

* The figures in parentheses indicate the final concentration of the substance in the complete reaction medium. This notation is used in all the tables.

The actively phosphorylating oxidation showed maintenance of the Δ7 P near the starting level. Omission of necessary components caused great decreases in Δ7 P. Maintenance of Δ7 P therefore represents a resultant between the action of phosphatases, ATPases, etc., on ATP and the synthesis of new phosphate bonds coupled to the oxidation. In the absence of the latter the level of Δ7 P falls to very low values.

The amount of inorganic P^{32} esterified in these experiments represents a semiquantitative measure of aerobic phosphorylation, as discussed in Paper I (3).

That the DPN added to these systems actually functions as a carrier (rather than some impurity contained in the preparations) is indicated by the fact that reduction of DPN, with the formation of the characteristic reduced band of $DPNH_2$ at 340 mμ, is readily observed when BOH, diluted enzyme, and DPN are incubated together, cytochrome oxidase being inhibited by 0.001 M cyanide. The additional presence of ATP or Mg^{++} has no effect on the rate of reduction of DPN in such experiments.[2]

TABLE II

Non-Reactivity of Acetoacetate

The system contained 0.005 M $MgSO_4$, 0.02 M glycylglycine buffer, pH 7.6, 0.02 M nicotinamide, 0.002 M ATP, 0.0003 M DPN, 10^{-5} M cytochrome *c*, 243,000 counts per minute of P^{32} as $HPO_4^=$, and 0.02 M *dl*-β-hydroxybutyrate or 0.01 M acetoacetate as indicated. 0.50 ml. of Enzyme II (in 0.15 M NaCl) in the side arm, tipped after 5 minutes equilibration into the main compartment to make a total volume of 2.0 ml. Time, 20 minutes.

Substrate	Acetoacetate		O_2 uptake	P^{32} esterified
	0 time	20 min.		
	c.mm.	*c.mm.*	*c.mm.*	*per cent*
None		0	4	1.78
β-Hydroxybutyrate	0	127	59	24.2
Acetoacetate		402	7	2.69
Same, 0 time	414			0.41

In order to demonstrate that the further oxidation of small amounts of acetoacetate does not cause significant phosphate esterification, acetoacetate and BOH were tested side by side in a series of enzyme preparations. Typical data from one of these experiments are given in Table II. It can be seen that acetoacetate by itself does not contribute significantly to oxygen uptake or P^{32} esterification. It may be concluded that further oxidation of acetoacetate may be excluded as being responsible for any significant amount of phosphate esterification and that the observed phosphorylation is due only to the DPN-linked oxidation.

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[2] Unpublished observations.

The P^{32} esterified in the complete system has been found to be precipitable to the extent of 90 per cent by barium acetate at pH 8.2. Furthermore, from 75 to 90 per cent of the esterified P^{32} can be converted into inorganic phosphate (precipitable by magnesia mixture) by a 10 minute period of hydrolysis in 1 N H_2SO_4 at 100°. These facts indicate that the esterified P^{32} is largely in the form of the easily hydrolyzable groups of ATP (they do not exclude the possibility that the esterified P^{32} may be in the form of inorganic pyrophosphate). It also appears unlikely from these data that the DPN molecule itself contains any substantial part of the esterified P^{32}. Labile phosphate esters of the type detectable by the Lowry and Lopez method (15) do not accumulate in these systems coupled to oxidation of BOH.

TABLE III

Specificity of Adenine Nucleotide As Phosphate Acceptor

The main compartment contained 0.005 M $MgSO_4$, 0.02 M nicotinamide, 0.02 M glycylglycine buffer, pH 7.8, 0.02 M β-hydroxybutyrate, 10^{-5} M cytochrome *c*, phosphate esters in the concentrations indicated, 278,000 counts per minute of P^{32} as $HPO_4^=$, 0.0005 M DPN. At zero time 0.50 ml. of Enzyme I was tipped into the main compartment to make a total volume of 2.0 ml. Time, 18 minutes.

Phosphate ester	Concentration	Acetoacetate formed	P^{32} esterified
	M	*c.mm.*	*per cent*
ATP	0.0016	61	37.0
ADP	0.0012	58	43.0
Muscle adenylic acid	0.002	54	4.0
Fructose-1,6-diphosphate	0.002	54	0.14
Inorganic pyrophosphate	0.002	46	0.56
α-Glycerophosphate	0.002	46	0.15
Thiamine pyrophosphate	0.002	43	0.14
Yeast adenylic acid	0.002	48	0.24

The adenosine polyphosphates are specific phosphate acceptors in this system. In Table III are shown data collected from experiments in which different phosphate esters were substituted for ATP. ATP and adenosine diphosphate (ADP) were equally effective. Muscle adenylic acid was slightly active.[3] Fructose-1,6-diphosphate, inorganic pyrophosphate, α-glycerophosphate, thiamine pyrophosphate, and yeast adenylic acid were ineffective as primary phosphate acceptors. It appears that ADP may be the primary acceptor in this phosphorylation, analogous to the rôle of ADP in triose phosphate oxidation (16).

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[3] Since this manuscript was prepared it has been found that muscle adenylic acid may serve as phosphate acceptor under special conditions and has actually been used in non-isotopic experiments to determine the P:O ratio of the reaction. The phosphorylation of adenylic acid is more labile to autolytic inactivation than is that of ADP.

It has been customary in studies of aerobic phosphorylation to use as secondary phosphate acceptors various substances such as creatine, glucose, glucose-6-phosphate, etc., which can be enzymatically phosphorylated by ATP. By using such acceptors it is often possible to trap esterified phosphate and cause a net accumulation of newly esterified phosphate coupled to oxidation. However, the preparations studied here are lacking in the necessary transphosphorylases which presumably have been removed in the isolation of the particulate material used as enzyme. For this reason it is necessary to measure esterification of inorganic phosphate by the use of P^{32} as a tracer, supplemented by data on the maintenance of the Δ7 P of ATP coupled to the oxidation. Owing to these technical difficulties it has not been possible to estimate accurately the efficiency of the coupled esterification by determination of the ratio of newly esterified phosphate to oxygen taken up in the methods used.[3]

Nicotinamide was used in the reaction medium in most of the experiments reported in this paper to inhibit destruction of DPN by nucleotidases (17). However, its presence was found not to be absolutely necessary and it was not used in later experiments.

Selective Inactivation of Phosphorylating Enzymes—The data already presented indicate that the esterification of inorganic phosphate coupled to BOH oxidation is not an essential part of the electron transport process, since the oxidation is not impaired by omitting those components necessary for phosphorylation (Mg^{++}, ATP).

In the course of these experiments it was found possible to inactivate selectively those enzymes necessary for the esterification reaction without impairing the enzymes involved in oxygen uptake simply by incubating the enzyme suspension at 30° for short periods of time before adding it to the reaction medium. In Table IV are collected the data of an experiment in which the enzyme was placed in the side arm of the Warburg vessels and incubated at 30° for various lengths of time before being tipped into the main compartment, which contained all the components necessary for oxidation and phosphorylation. Incubation of the enzyme under these circumstances for a period of 10 minutes caused no change in rate of oxidation or extent of phosphorylation. However, incubation for 30 minutes or longer caused the esterification reaction to disappear without impairing the oxidation. In general, a 5 minute period of incubation of the enzyme in the side arm for temperature equilibration was found to be a safe procedure, causing no significant loss of activity in several experiments.

The stability of the enzymes involved in the DPN-linked oxidation and the lability of the phosphorylating system can also be demonstrated by aging of the enzyme preparation at 2°. The oxidative system loses little if any activity in 3 to 4 days at 2°; the phosphorylating activity is lost after 12 to 24 hours.

These experiments indicate strongly that the phosphorylation process is not obligatory to electron transport and may be dissociated or "decoupled" from the oxidation by simply omitting Mg^{++} or ATP or by selective inactivation.

We have not succeeded in restoring the activity of an inactivated enzyme preparation by addition of known coenzymes, etc., or by a boiled extract of a fresh, active enzyme preparation.

Decoupling of Phosphorylation by Inhibitors—The esterification of phosphate may be decoupled from the oxidation not only by omitting Mg^{++} or ATP and by selective inactivation but also by the use of certain inhibitors. In Table V are shown data collected from several experiments in which the effect of various substances on the oxidation and phosphorylation was examined.

TABLE IV

Selective Inactivation of Phosphorylating Enzymes

The main compartment contained 0.005 M $MgSO_4$, 0.02 M glycylglycine buffer, pH 7.5, 0.02 M nicotinamide, 0.0005 M DPN, 0.0018 M ATP, 0.02 M β-hydroxybutyrate, 10^{-5} M cytochrome *c*, 308,300 counts per minute of P^{32} as $HPO_4^{=}$. The side arm contained 0.50 ml. of Enzyme II suspension in H_2O, tipped in at the time intervals indicated below to make a total volume of 2.0 ml. Each flask was then incubated 25 minutes after mixing.

Incubation period at 30° prior to addition	O_2 uptake	Acetoacetate formed	$\Delta 7$ P	P^{32} esterified
min.	*c.mm.*	*c.mm.*	γ	*per cent*
0	65	141	174	34.0
5	64	132	189	36.3
10	64	133	194	35.0
30	81	151	46	1.6
60	72	139	34	1.2
Reaction stopped at 0 time			216	0.4

Arsenite, 2,4-dinitrophenol, *p*-nitrophenol, Ca^{++}, and $SO_4^{=}$ were found to be potent inhibitors of the coupled phosphorylation, showing essentially no effect on the oxidation. Arsenate, pyrophosphate, and octanoate were somewhat less effective as decoupling agents. Fluoride caused some inhibition of oxidation but a great increase in amount of P^{32} esterified, an effect which might have been predicted owing to the well known inhibition of phosphatases by fluoride. Phlorhizin, iodoacetate, ethyl carbamate, and malonate did not inhibit the phosphorylation in the concentrations tested, although malonate inhibited the oxidation of BOH somewhat.

The decoupling of phosphorylation by arsenite is of special interest. It may possibly explain the failure of Ochoa (18) to realize oxidative phosphorylation coupled to the TPN-linked oxidation of isocitrate to oxalosuccinate in pig heart preparations supplemented with ATP and Mg^{++}, which were otherwise readily capable of oxidative phosphorylation during α-ketoglutarate oxidation. In the experiments on isocitrate oxidation Ochoa prevented further oxidation of α-ketoglutarate, which would have complicated the findings, by the use of arsenite as inhibitor. By analogy with the DPN system studied here it is possible that the arsenite used may have decoupled whatever phosphorylation accompanied this oxidation.

Table V

Decoupling of Phosphate Esterification by Inhibitors

The data were obtained from several sets of experiments arranged as in the previous tables with the inhibitors added in the concentrations listed. Control values (no inhibitor present) are given with each set in bold-faced type.

Experiment No.	Inhibitor	Concentration	O_2 uptake	Acetoacetate formed	P^{32} esterified
		M	*c.mm.*	*c.mm.*	*per cent*
1	None		52	116	**16.1**
	Arsenate	0.005	56	112	8.3
	Malonate	0.0075	27	41	10.1
2	None		82	151	**54.5**
	2,4-Dinitrophenol	0.0002	70	127	5.3
	$CaCl_2$	0.005	68	145	2.6
	Phlorhizin	0.0002	75	121	61.0
	Fluoride	0.018	48	110	81.5
	Iodoacetate	0.0025	60	117	55.3
	p-Nitrophenol	0.0002	78	148	7.6
	Pyrophosphate	0.005	72	157	25.2
3	None		88	186	**21.6**
	Arsenite	0.009	88	182	0.7
4	None			197	**27.6**
	Ethyl carbamate	0.0005		191	35.4
5	None		112	218	**36.2**
	Octanoate	0.008	78	164	7.8
6	None		43	92	**36.5**
	NaCl	0.02	45	89	33.5
	Na_2SO_4	0.02	48	100	3.4
	NH_4Cl	0.02	46	88	37.0
	$(NH_4)_2SO_4$	0.02	46	96	3.3

Decoupling of oxidative phosphorylation by 2,4-dinitrophenol has been observed by Loomis and Lipmann (19) and by Hotchkiss (20). 2,4-Dinitrophenol was the most effective decoupling agent for the system studied here, decoupling effects being detectable at concentrations as low as 10^{-6} M. The effect of Ca^{++} is possibly related to the activating effect of Ca^{++} on liver ATPase (*cf.* (21)). The lack of any effect of phlorhizin on the phosphorylation is noteworthy.

Inhibition of Esterification by DPN—In Paper I (3) it was shown that the aerobic incubation of high concentrations of $DPNH_2$ with the enzyme system caused esterification of inorganic P^{32} under appropriate conditions. These findings appear to be at variance with unpublished data quoted by Ochoa (4) which showed no net esterification of inorganic phosphate when high concentrations of $DPNH_2$ were incubated with heart preparations under conditions comparable to those used in our study. It appeared to us that these outwardly contradictory findings could be explained by an inhibitory action of high concentrations of DPN (or $DPNH_2$) which could cause inhibition of the esterification to such a degree as to show no net phosphorylation but an amount of turnover still detectable by the tracer technique. To test this explanation DPN concentration was varied from 0.0001 to 0.005 M in the standard BOH system (Table VI). It is clearly evident that the higher concentrations of DPN are highly inhibitory to phosphate esterification. It appears possible, from consideration of other data, that $DPNH_2$ as such is less inhibitory than DPN, although this suggestion is difficult to test. It appears likely that the inhibitory action of high concentrations of DPN is actually due to the DPN rather than to impurities contained in it, since DPN inactivated by alkali shows much less inhibition than does untreated DPN in a system containing low concentrations of the intact nucleotide. These findings probably furnish a partial explanation for Ochoa's failure to obtain net synthesis of phosphate

TABLE VI

Effect of DPN Concentration on Coupled Esterification

The main compartment contained 0.005 M $MgSO_4$, 0.02 M glycylglycine buffer, pH 7.5, 0.02 M nicotinamide, DPN in the concentrations indicated, 0.0018 M ATP, 0.02 M BOH, 10^{-5} M cytochrome *c*, 308,300 counts per minute of P^{32} as $HPO_4^=$. The side arm contained 0.50 ml. of Enzyme I suspension. Time, 15 minutes.

DPN concentration	O_2 uptake	Acetoacetate formation	P^{32} esterified
M	*c.mm.*	*c.mm.*	*per cent*
0.0001	64	148	15.1
0.0003	84	164	20.4
0.0007	80	160	21.6
0.0015	76	165	17.3
0.0030	82	170	6.4
0.0050	85	161	2.3

bonds coupled to the oxidation of high concentrations of $DPNH_2$. The degree of inhibition produced, however, still allows detection of turnover with the use of P^{32}. Owing to this effect of DPN, optimal phosphorylation activity is usually observed at DPN concentrations somewhat below optimal concentrations for maximum oxidation rates.

Dependence of Coupled Phosphorylation on Tonicity of Medium—In a study of the requirements of the fatty acid oxidase system, it was found that the oxidation of octanoate, pyruvate, or malate in particulate fractions of rat liver required approximately isotonic reaction media and it was shown that either electrolytes, such as NaCl or LiCl, or non-electrolytes, such as sucrose or glucose, sufficed to meet the requirement (11). In the light of other investigations in this laboratory (22) it appears that this requirement may be due to the fact that the oxidations described take place in the mitochondria, which are known to be morphologically sensitive to tonicity of the medium. Since the phosphorylating oxidation under study occurs in preparations of mitochondria, an experiment designed to test the effect of tonicity was performed. The ionic strength of the test system was reduced as far as possible without impairing the oxidation by reducing the concentration of buffer and substrate, by omitting nicotinamide, and by suspending the enzyme in H_2O instead of saline. This served as a "hypotonic" control. In other vessels similarly arranged KCl, NaCl, or sucrose was added in amounts sufficient to approximate osmolarity. The effects of these changes on the coupled esterification are shown in Table VII.

TABLE VII

Effect of Tonicity on Coupled Phosphorylation

The main compartment contained 0.10 ml. of $MgSO_4$ (0.002 M), 0.05 ml. of glycylglycine buffer, pH 7.5 (0.005 M), 0.40 ml. of ATP (0.002 M), 0.20 ml. of cytochrome *c* (10^{-5} M), 0.05 ml. BOH (0.01 M), 0.10 ml. of P^{32} (162,500 counts), 0.10 ml. of DPN (0.0005 M), 0.50 ml. of Enzyme I in water. Total volume, 2.0 ml. Addition of salts and sucrose as indicated. Time, 25 minutes.

Solute added	Oxygen uptake	Acetoacetate formed	P^{32} esterified
	c.mm.	*c.mm.*	*per cent*
None	72	149	5.7
"	74	148	7.7
0.08 M KCl	65	134	48.2
0.08 " NaCl	67	130	51.0
0.08 " sucrose	64	130	51.8
0.15 " "	65	138	50.1

It can be seen from the data that the oxidation of BOH is virtually independent of tonicity. However, the coupled esterification is dependent on the tonicity of the medium, as are the complex oxidations of fatty acid or pyruvate in such preparations. It should be stressed that the requirement of solutes in approximately osmolar concentrations in this system and in the fatty acid oxidase system should not be interpreted simply and solely on the basis of osmotic effects, since the character of the solute affects the condition of the mitochondria in other ways. For instance, in sucrose solutions the mitochondria are discrete and rod-shaped, whereas in isotonic saline they tend to agglutinate (12). This solute effect is evident also in the experiments of Potter *et al.* (23).

Site of Coupling Reaction in Electron Transport Chain—In the experiments described the enzyme system causes electron transport from BOH to oxygen. In Paper I (3) it was shown that esterification occurred during electron transport from $DPNH_2$ to oxygen. It might be concluded that the esterification noted with the BOH system studied here occurs only during electron transport between $DPNH_2$ and oxygen. On thermodynamic grounds esterification of phosphate during the interaction of BOH with DPN is unlikely, since the standard oxidation-reduction potential of the BOH-dehydrogenase system is −0.293 volt (24) and that of DPN-$DPNH_2$ is approximately −0.28 volt (25) at pH 7.0. Participation of inorganic phosphate in this reaction has not been observed[2] (*cf.* (7, 24)).

No esterification of phosphate could be observed coupled to a DPN-linked oxidation-reduction. The oxidation of BOH was allowed to occur anaerobically with oxalacetate as electron acceptor rather than the cytochrome system. The enzyme preparations contain considerable malic dehydrogenase reactive with DPN. In the presence of the two dehydrogenases, then, BOH will be oxidized by oxalacetate via DPN and acetoacetate and *l*-malate would be the expected end-products. The potential of the malate-oxalacetate system is such as to cause the reaction to proceed predominantly toward reduction of oxalacetate. In Table VIII are shown data on this experiment. An aerobic control experiment with BOH alone as substrate showed the usual esterification. However, when BOH was oxidized by oxalacetate anaerobically, with all necessary components present and with about the same amount of electron turnover, as indicated by acetoacetate formation, no esterification occurred. Therefore, the DPN-linked oxidation-reduction,

$$\text{(1)} \qquad l\text{-}\beta\text{-Hydroxybutyrate} + \text{oxalacetate} \rightleftharpoons \text{acetoacetate} + l\text{-malate}$$

does not cause esterification of phosphate.

The most decisive experiment to rule out interaction of BOH with DPN as the site of an esterification of phosphate would be to allow BOH to reduce DPN anaerobically in the presence of the components necessary for phosphorylation without further reaction of the $DPNH_2$ formed. Such an experiment requires the use of high concentrations of DPN as acceptor in order to obtain sufficient electron turnover, comparable to that usually realized in the aerobic system. Since high concentrations of DPN inhibit the over-all reaction, as already shown, it is obvious that a completely decisive experiment might be difficult to arrange. Several experiments of this type showed no esterification of P^{32} under the conditions outlined. Aerobic controls with identical levels of DPN showed some esterification, although the amount of esterification in these controls was quite low.

The rôle of the cytochrome system in the esterification reaction was then examined in an effort to determine whether a functioning cytochrome system is essential in the esterification of phosphate coupled to electron transport between $DPNH_2$ and oxygen. In the first type of experiment electron transport from the BOH system to oxygen was shunted away from the cytochrome system by omitting cytochrome *c*, which is otherwise essential for electron transport, and causing the reaction with molecular oxygen to occur through the use of the autoxidizable carriers methylene blue and brilliant cresyl blue. These dyes are perfectly capable of substituting for the cytochrome system as far as oxidation of BOH is concerned. However, as may be seen in the data of an experiment in Table IX, electron transport through the artificial carriers instead of the cytochrome system causes a complete failure of the coupled esterification to occur. These data would therefore imply that a functioning cytochrome system is involved in the coupled phosphorylation. However, it should be mentioned that if both cytochrome *c* and either methylene blue or brilliant cresyl blue are present together no esterification occurs. Under these circumstances

TABLE VIII

Absence of Esterification during Anaerobic Oxidation-Reduction between β-Hydroxybutyrate and Oxalacetate

The main compartment contained 0.005 M $MgSO_4$, 0.02 M glycylglycine buffer, pH 7.6, 0.02 M nicotinamide, 0.0005 M DPN, 0.02 M *dl*-β-hydroxybutyrate, and 0.01 M oxalacetate as indicated below, 103,700 counts per minute of P^{32} as $HPO_4^{=}$, 0.0013 M ATP, 10^{-5} M cytochrome *c*. The side arm contained 0.50 ml. of Enzyme I tipped in after 5 minutes equilibration with the gas specified. Time, 30 minutes.

Substrate	Gas phase	O_2 uptake	Acetoacetate formed	P^{32} esterified
		c.mm.	*c.mm.*	*per cent*
BOH	O_2	40	76	17.7
None	"	4	4	2.9
BOH	N_2		8	1.2
Oxalacetate	"		4	1.3
BOH + oxalacetate	"		70	1.7

it is impossible to determine whether the dye is preferentially used as carrier instead of cytochrome, thereby causing non-phosphorylative oxidation, or whether the effect of the dye is to act as an inhibitor or decoupling agent, perhaps in the same way as 2,4-dinitrophenol, in addition to serving as an electron carrier. Therefore the cytochrome system was substituted with an entirely different type of acceptor, namely ferricyanide.

TABLE IX

Requirement of Cytochrome System for Esterification Reaction

The main compartment contained 0.005 M $MgSO_4$, 0.02 M nicotinamide, 0.02 M glycylglycine buffer, pH 7.8, 0.02 M β-hydroxybutyrate, 0.0005 M DPN, 10^{-5} M cytochrome *c*, 366,600 counts per minute of P^{32} as $HPO_4^=$, 0.0015 M ATP, dyes in the concentrations stated below. 0.6 ml. of Enzyme I added to the main compartment at zero time to make a total volume of 2.0 ml. Time, 32 minutes.

Carrier	O_2 uptake	Acetoacetate formed	P^{32} esterified
	c.mm.	*c.mm.*	*per cent*
None	4	4	1.22
Cytochrome *c*	54	116	16.1
0.00025 M methylene blue	58	124	1.14
0.0005 " brilliant cresyl blue	54	122	0.44

TABLE X

Requirement of Cytochrome System for Esterification Reaction

The main compartment contained 0.10 ml. of $MgSO_4$ (0.005 M), 0.10 ml. of glycylglycine buffer, pH 7.4 (0.02 M) in the aerobic experiment or 0.10 ml. of $NaHCO_3$ (0.025 M) in the anaerobic experiment, 0.10 ml. of nicotinamide (0.01 M), 0.50 ml. of ATP (0.002 M), 0.10 ml. of DPN (0.0005 M), 0.20 ml. of cytochrome *c* (2×10^{-5} M) in the aerobic experiment (omitted in the anaerobic experiment and replaced by 0.20 ml. of $K_3Fe(CN)_6$, 0.02 M), 0.10 ml. of BOH (0.02 M), 0.10 ml. of inorganic phosphate labeled with P^{32} = 340,500 counts, and water. Enzyme I (0.50 ml.) tipped into the main compartment after a temperature equilibration of 5 minutes. Total volume, 2.0 ml. Time, 18 minutes. Gas phases as indicated.

Substrate	Gas phase	Acceptor	O_2 uptake	Acetoacetate formed	P^{32} esterified
			c.mm.	*c.mm.*	*per cent*
None	Air	Oxygen	2	1	0.90
BOH	"	"	68	147	28.5
None	95% N_2-5% CO_2	$K_3Fe(CN)_6$	36*	2	0.55
BOH	95% " 5% "	"	190*	133	0.37

* These figures represent CO_2 liberated due to reduction of $K_3Fe(CN)_6$. They are not corrected for retention.

In Table X are shown the data of an experiment in which the standard aerobic system was compared with a similarly arranged system containing all components except cytochrome *c*, which was replaced by ferricyanide and bicarbonate buffer. Reduction of ferricyanide was measured manometrically (26). Acetoacetate formation was determined manometrically (27) after first liberating CO_2 from the bicarbonate buffer by short incubation at pH 4.5. This was necessary, since ferricyanide interfered with the colorimetric method ordinarily used. As can be seen from the data, the control aerobic system showed the expected esterification. However, when ferricyanide was used as acceptor, no esterification occurred despite the fact that oxidation of BOH occurred at about the same rate in both the aerobic and anaerobic systems, as evidenced by the yields of acetoacetate.

In view of these data it may be tentatively concluded that an actively functioning cytochrome system plays an integral rôle in the enzyme system, causing esterification of phosphate coupled to the type of electron transport studied.

In Paper I (3) it was shown that no esterification of phosphate occurred during electron transport between $DPNH_2$ and high concentrations of cytochrome *c* under anaerobic conditions or when cytochrome oxidase was inhibited by cyanide. This type of experiment was repeated with the BOH system as electron donor and high concentrations (0.002 M) of cytochrome *c* as acceptor. In such experiments it was felt that measurement of acetoacetate formed would provide an indication of the magnitude of electron turnover and therefore would be better suited for this test. However, the presence of such high concentrations of cytochrome *c* caused some complications which did not permit a completely decisive experiment. As was shown before, high concentrations of cytochrome *c* inhibited the phosphorylation considerably. In addition, the BOH dehydrogenase system was also strongly inhibited. With such extensive inhibitions it is likely that the action of phosphatases on ATP would far outstrip the rate of esterification. It must be recalled that a sufficiently high concentration of cytochrome *c* to provide substantial electron turnover involves the presence of a very high concentration of the protein (0.004 M cytochrome *c* represents a concentration of about 5.4 per cent of the pure protein). Nonspecific protein interactions may be responsible for the large inhibitions observed. At any rate no significant incorporation of P^{32} occurred in several experiments. It would appear from our experience that demonstration of the esterification reaction between $DPNH_2$ and cytochrome *c* offers practical difficulties.

In Paper I experiments were described in which it was attempted to localize at least part of the esterification in electron transport between cytochrome *c* and oxygen by using cysteine, hydroquinone, ascorbic acid, etc., as non-enzymatic reductants of cytochrome *c* without finding any esterification except with ascorbic acid, which may be a special case. These experiments have been repeated under a variety of conditions without any significant positive results. It is felt that these experiments have some validity in ruling out this phase of electron transport as a site of phosphate esterification, since cysteine, at least, does not inhibit the normal esterification coupled to BOH oxidation. An inhibitory or decoupling action of cysteine may therefore be excluded as explaining negative results when it is tested alone as a reductant of cytochrome *c*.

DISCUSSION

The data reported in these two papers demonstrate quite conclusively that esterification of inorganic phosphate is coupled to electron transport between $DPNH_2$ and oxygen in the system studied. These findings are not only compatible with thermodynamic analysis of observed efficiency data on oxidative phosphorylation but also provide a more comfortable distribution of phosphorylative loci within the Krebs cycle, in the light of the comments made in the introduction of Paper I (3) and the analysis of Ogston and Smithies (28). It would appear likely that, by analogy, TPN-linked oxidations may also be accompanied by phosphorylation. Work on this question is in progress.

The efficiency of the DPN-linked phosphorylation in the conversion of energy yielded on oxidation into phosphate bond energy has not been considered in this paper. Obviously it will be important to know whether one, two, or more esterifications are coupled to this oxidation in approaching the mechanism experimentally and work on this question is in progress.

The attempts described to establish whether esterification of phosphate occurs between $DPNH_2$ and cytochrome *c* and between cytochrome *c* and oxygen have yielded no decisive answer. Although it would appear that the failure to observe esterification of phosphate in the latter case, when cysteine was used as a non-enzymatic reductant of cytochrome *c*, was decisive, it can be argued that the activity of the enzymes responsible for such an esterification may be conditioned by phosphorylations taking place at some point in the electron transport chain prior to this phase. In any event, at this stage of our knowledge of such systems it would be unwise to accept negative results unconditionally. In the case of the experiments on phosphorylation coupled to electron transport between $DPNH_2$ and cytochrome *c* (3), which must be acknowledged as the most likely site of a phosphorylation, the results have been negative despite the fact that the conditions of these anaerobic experiments were such as to be quite comparable to control aerobic experiments as far as electron turnover was concerned. Here again some special condition of the experiments may be responsible for the failure to detect phosphorylation. For instance it may well be that the phosphorylation requires the presence of molecular oxygen *per se*, not purely as an ultimate electron acceptor, but also to condition the activity of an essential catalyst. The question of the function of cytochromes *a* and *b* in electron transport comes to mind in this connection. Such a situation is also reminiscent of the findings of Stern and Melnick on the control of glycolysis in retina by an iron porphyrin catalyst (29) and the observations of Laser on the sensitivity of glycolysis in tissue slices to oxygen tension (30).

The data presented also raise some questions concerning the mechanism of electron transport between $DPNH_2$ and oxygen: The existence of TPN-cytochrome *c* reductase, a flavoprotein found in yeast catalyzing the reduction of cytochrome *c* by $TPNH_2$ (31), has focused attention on the possibility that a similar flavoprotein is responsible for the analogous reduction of cytochrome *c* by DPN. Evidence for the existence of such an enzyme has been presented by Lockhart and Potter (32) and by Altschul, Persky, and Hogness (33). In addition Potter has devised an assay method for the enzymes responsible for electron transport between DPN and cytochrome *c* (14).

However, it is rather difficult to reconcile such a simple, one-stage, non-phosphorylating electron carrier between DPN and cytochrome *c* with the observations we have reported. Our data indicate that two possibilities for this link are open. First, it is possible that there are actually two pathways of electron transport between DPN and cytochrome *c*, one of which is non-phosphorylative and which does not require inorganic phosphate, ATP, or Mg^{++}. The second would be a pathway which does require the above factors and to which esterification of phosphate is coupled. The second pathway is labile to aging (probably enzymatic destruction of an unknown essential component) and when it is thus inactivated all the electron transport may be handled by the first pathway, which might well involve a simple flavoprotein carrier analogous to TPN-cytochrome *c* reductase. An alternative explanation is that there is but one catalyst which may be phosphorylative or non-phosphorylative, depending on the presence and concentration of components necessary for phosphorylation. Since the esterification process must be simultaneous with the act of oxidation, there are only limited reaction possibilities. One might be mentioned. If a flavin prosthetic group is the actual electron carrier for both processes, in a non-phosphorylating electron transport reduction of the conjugated double bond system yields the leuco form which is reversibly oxidized to regenerate the oxidized form. In a phosphorylative transport of electrons the same molecule may be reduced and reoxidized by a different mechanism, possibly involving one or both of the carbonyl groups of the isoalloxazine ring as well as inorganic phosphate. The carbonyl groups deserve special attention by analogy with the rôle of carbonyl group oxidation in the esterification of phosphate coupled to the oxidation of glyceraldehyde phosphate.

A mechanism involving DPN as phosphate acceptor during the reaction between $DPNH_2$ and the next carrier should not be overlooked in view of the known conversion of DPN into TPN in the presence of ATP (34, 35). Lipmann (5) and Ogston and Smithies (28) have discussed such energy "transformers" in general terms.

SUMMARY

The oxidation of β-hydroxybutyrate to acetoacetate by molecular oxygen in a particulate fraction of rat liver requires the presence of diphosphopyridine nucleotide and cytochrome *c*. No esterification of inorganic phosphate occurs under these conditions. However, when adenosine triphosphate and Mg^{++}, which have no effect on the rate of oxidation, are also present, then inorganic phosphate labeled with P^{32} is incorporated into an esterified form having the characteristics of the easily hydrolyzable phosphate groups of ATP. This esterification is coupled to the oxidation. Acetoacetate oxidation is excluded as a factor in the esterification, which is therefore coupled only to the DPN-linked oxidation. ADP appears to be the specific phosphate acceptor. The enzymes responsible for the coupled esterification may be selectively inactivated or inhibited by certain compounds without affecting the oxidation itself. No esterification accompanies the interaction of β-hydroxybutyrate with oxalacetate via DPN. The cytochrome system is obligatory for the esterification accompanying BOH oxidation, since its substitution by artificial electron carriers or acceptors causes the esterification reaction to be lost. Experimental attempts to localize the sites of esterification more closely have not given definitive answers.

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STRUCTURAL REQUIREMENTS FOR SPECIFIC INHIBITORS OF CARBOXYPEPTIDASE*

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In a recent investigation (1) it was shown that the optical specificity of specific inhibitors of carboxypeptidase is opposite in sign to that of specific substrates. Thus D-phenylalanine is strongly inhibitory, whereas carbobenzoxyglycyl-D-phenylalanine (CGP) is neither inhibitory nor hydrolyzable (2, 3). Conversely, L-phenylalanine is not inhibitory, whereas L-CGP is the most readily hydrolyzable substrate for carboxypeptidase (4, 5).

The present investigation was undertaken in an effort to determine the origin of this apparent paradox. In particular, it was desired to determine the effect of a free α-amino group and of other structural elements of D-phenylalanine on inhibition of the hydrolysis of L-CGP by carboxypeptidase. The evaluation of the results was predicated on the assumption that only those compounds are specific inhibitors for carboxypeptidase which, by combining with the same active centers on the enzyme surface as does the specific substrate (L-CGP), produce *competitive* inhibition (6).

EXPERIMENTAL

Enzyme—Six times recrystallized carboxypeptidase was prepared as previously described (2, 7).

Substrate and Inhibitors—The preparation of carbobenzoxyglycyl-L-phenylalanine (CGP) has already been described (2).

N-Methyl-DL-phenylalanine and N,N-dimethyl-DL-phenylalanine were received from Dr. P. Handler. Both compounds had correct N contents. Carbobenzoxyglycine was synthesized in this laboratory by Mr. J. E. Snoke (8); carbobenzoxyglycylglycine was received from Dr. J. S. Fruton. β-Phenylbutyric acid was synthesized according to standard procedures (9). DL-α-Phenylethylamine, β-phenylethylamine, phenylacetic acid, *p*-

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nitrophenylacetic acid, hydrocinnamic acid, butyric acid, propionic acid, chloroacetic acid, and benzoic acid were Eastman preparations.

Methods—Enzymatic measurements were carried out at 25° in a 0.04 M phosphate buffer, pH 7.5, containing 0.1 M LiCl. Enzyme solutions were prepared immediately[1] before the start of the enzymatic experiments from a stock solution containing about 0.2 to 0.5 mg. of N per cc. The latter was prepared about every 3rd day by dissolving a stock suspension of crystals in 10 per cent LiCl. Calculated amounts of substrate (CGP) and inhibitor, respectively, were dissolved in buffer, adjusted at the glass electrode (Beckman) to pH 7.5, and diluted with buffer to the proper volume. Substrate and inhibitor solutions were then mixed and the enzyme solution added at zero time. The concentration of carboxypeptidase in the final mixture was such as to cause more than 60 per cent hydrolysis within 120 minutes. The colorimetric ninhydrin method (11) as described by Schwert (12)[2] was used to follow the course of reaction. Each inhibitor was tested for possible interference with the color reaction. Only DL-α-phenylethylamine and β-phenylethylamine produced a color which was corrected for in the final readings.

Results

Lineweaver and Burk (6) have shown that the following equation applies to the competitive inhibition of enzymatic reactions:

$$\frac{1}{v} = \frac{1}{V_{\text{max.}}}\left(K_m + \frac{K_m(I)}{K_I}\right)\frac{1}{a} + \frac{1}{V_{\text{max.}}} \qquad (1)$$

Here, v is the initial reaction velocity, and $V_{\text{max.}}$ the maximum reaction velocity. K_m is the enzyme-substrate dissociation constant, (I) the inibitor concentration, K_I the enzyme-inhibitor dissociation constant, and the initial substrate concentration, all in moles per liter. In the case of competitive inhibition (1), a plot of $1/v$ against $1/a$ yields straight lines which intersect at a common ordinate intercept, provided (I) remains constant as a is varied. The increase in slope is a function of (I) and $1/K_I$. At equal concentrations of two inhibitors, the slope will be higher for the more potent inhibitor (higher $1/K_I$).

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[1] Smith and Hanson (10) (private communication from Dr. Emil Smith) recently obtained evidence that crystalline carboxypeptidase is inhibited by orthophosphate, pyrophosphate, and other inorganic salts which combine with metals, notably with magnesium, which is probably an integral part of the enzyme molecule. Since inhibition by orthophosphate is a slow reaction, requiring several hours for completion and amounting to about 10 per cent in the presence of 0.01 M phosphate and to about 60 per cent in 0.1 M phosphate, it is unlikely that this type of inhibition played a significant rôle in the present measurements or in those previously reported (1, 2, 7). Thus all measurements were performed in the presence of 0.04 M phosphate, and phosphate was withheld from the enzyme solution until immediately prior to the start of the enzymatic analyses which were concluded within about 120 minutes. Moreover, in the present work, determination of the extent of inhibition was based on initial velocity rates (30 minutes).

[2] Although this method requires empirical correction for the color loss due to oxidation, it has the practical advantage over the more precise method of Moore and Stein (13) that ammonia does not produce appreciable color interference with the determination of the free amino acid.

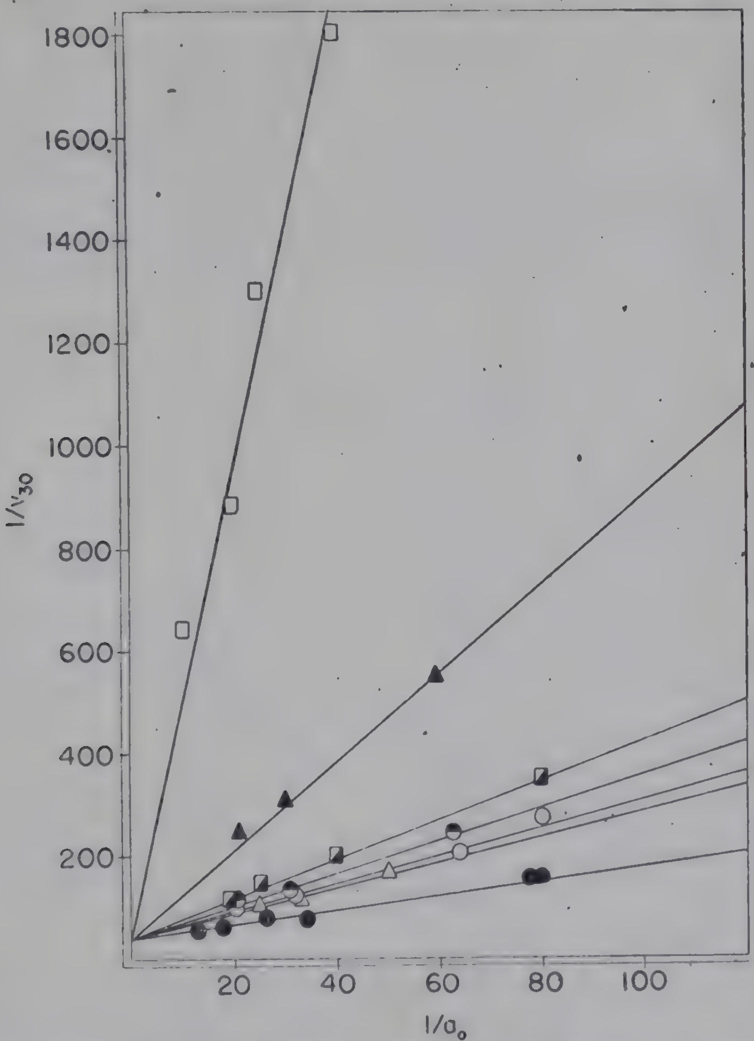

FIG. 1. Competitive inhibition by various compounds of the hydrolysis of L-CGP by carboxypeptidase. $1/v_{30}$ is plotted along the axis of the ordinate, and $1/a_0$ along the axis of the abscissa, according to text equation (1), where v_{30} is the moles per liter of substrate hydrolyzed in 30 minutes by 4×10^{-4} mg. of enzyme N per cc. a_0 is the initial substrate concentration. The straight lines are defined by the following symbols: ● = no added inhibitor; △ = 0.002 M *p*-nitrophenyl acetate; ○ = 0.010 M D-histidine; ◓ = 0.0025 M D-phenylalanine; ◩ = 0.002 M phenyl butyrate; ▲ = 0.002 M phenyl acetate; □ = 0.002 M hydrocinnamate. For K_I values, see Table I.

For purposes of presentation and discussion, the inhibitors which have been studied are divided into three groups of which the first and second cause strictly competitive and non-competitive inhibition, respectively.

TABLE I

Enzyme-Inhibitor Dissociation Constants (K_I) of Competitive Inhibitors of Carboxypeptidase

Inhibitor	K_I	Inhibitor	K_I
	10^{-3} M		10^{-3} M
D-Phenylalanine*†	2.0	β-Phenylbutyric acid	1.13
D-Histidine*	20.0	Phenylacetic acid	0.39
Hydrocinnamic acid	0.062	*p*-Nitrophenylacetic acid	2.5

* See Elkins-Kaufman and Neurath (1).

† This value was correctly stated in the text but erroneously given in Table I of that paper as 0.33×10^{-3}.

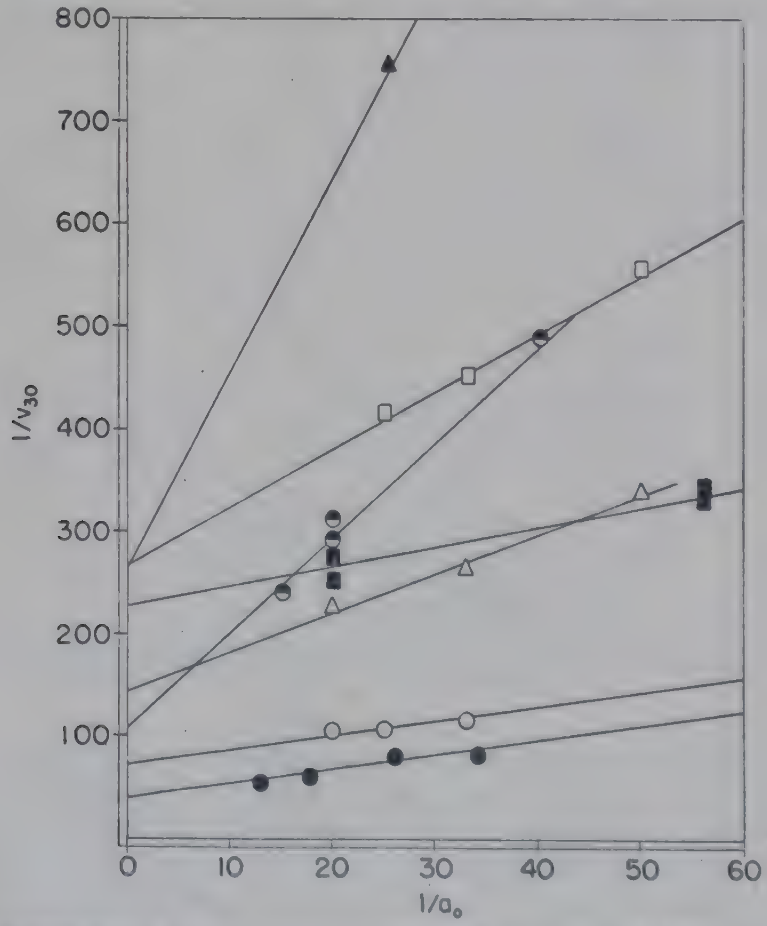

FIG. 2. Indeterminate and non-competitive (benzoate) inhibition, respective by various compounds of the hydrolysis of L-CGP by carboxypeptidase. For an e planation of the coordinates, see the legend to Fig. 1. The straight lines are defin by the following symbols: ● = no added inhibitor; ○ = 0.025 M chloroacetate; ◓ 0.05 M chloroacetate; △ = 0.001 M butyrate; ▲ = 0.005 M butyrate (this line is det mined by two additional points which fall outside this graph. They are defined the ordinates of 880 and 1230, corresponding to the abscissas of 33 and 50, resp tively); □ = 0.05 M propionate; ■ = 0.05 M benzoate (non-competitive inhibitio

Inhibition caused by the members of the third group was of an indete minate type.

Competitive Inhibitors—In addition to D-phenylalanine and D-histidine,
eviously reported (1), the following compounds were found to cause com-
titive inhibition of the system carboxypeptidase-L-CGP: hydrocinnamic
id, β-phenylbutyric acid, phenylacetic acid, and *p*-nitrophenylacetic
id. This is evidenced by the relative positions of the linear relations
own in Fig. 1. From these graphs, enzyme-inhibitor dissociation con-
ants, K_I, were calculated according to equation (1). The results are
ven in Table I in which, for comparison, analogous data previously ob-
ined for D-phenylalanine and D-histidine are likewise included.

Non-Competitive Inhibitors—Of the compounds tested in this work, only
enzoic acid was found to cause non-competitive inhibition. In the pres-

TABLE II

Compounds Which Fail to Cause Demonstrable Inhibition of System Carboxypeptidase-L-CGP

Compound	Concentration	Substrate concentration	$C_{obs.}$*	$C_{theor.}$*
	10^{-2} M	*10^{-2} M*		
-Methyl-DL-phenylalanine	2.54	2.58	19.5	19.7
,N-Dimethyl-DL-phenylalanine	2.50	2.67	20.6	20.0
L-α-Phenylethylamine	2.50	3.00	18.5	18.0
-Phenylethylamine	2.50	3.00	17.0	18.0
ippuric acid	5.00	2.37	24.2	21.5
" "	5.00	3.00	17.8	18.0
arbobenzoxyglycine	3.70	1.25	27.5	27.5
arbobenzoxyglycylglycine	5.00	4.00	15.7	14.5
"	4.00	4.00	13.5	14.5
"	3.34	3.00	19.0	18.0

* $C_{theor.}$ denotes the proteolytic coefficients for the hydrolysis of L-CGP without
dded inhibitor, and $C_{obs.}$ those determined in the presence of the compounds tested
or inhibitory activity. The latter were determined from the initially linear course
f the curves obtained when the data were plotted according to first order reaction
quations. $C_{theor.}$ was determined by interpolation of the graph shown in Fig. 3
1).

nce of 0.05 M sodium benzoate, the maximum velocity of the hydrolysis
f L-CGP, $V_{max.}$, was decreased 5.5-fold. The results are included in
ig. 2.

Indeterminate Inhibition—Inhibition caused by butyric acid, propionic
cid, and chloroacetic acid was of an indeterminate type. Although, as
hown in Fig. 2, linear relations were obtained when, for each inhibitor
oncentration, $1/v$ was plotted against $1/a$, both the slope and the ordinate
ntercept were changed as compared to the control measurements (L-CGP
lone). It may be significant that in relatively low inhibitor concentra-
ions the linear plots tended to be more nearly parallel to that obtained for

the hydrolysis of L-CGP alone (control), whereas in relatively higher c centrations they tended to affect the slope as well. This is true for buty acid and chloroacetic acid, which were tested in two concentrations, a suggests an approach to competitive inhibition in higher concentratio

Non-Inhibitors—Mono- or disubstitution of methyl groups into L phenylalanine abolishes completely the inhibitory activity of the pare D-amino acid. Since hippuryl-L-phenylalanine is a potent substrate f carboxypeptidase,[3] it was important to find that both reaction produc *i.e.* L-phenylalanine (1) and hippuric acid (Table II), are non-inhibito Carbobenzoxyglycine and carbobenzoxyglycylglycine are entirely devoid inhibitory activity. β-Phenylethylamine, the decarboxylated analog of phenylalanine, and its α isomer have no effect on the enzymatic activi of carboxypeptidase.

DISCUSSION

The present results afford a correlation of the inhibitory activity of t compounds that have been tested with the structural requirements f specific combination with carboxypeptidase. Such an analysis is limite however, by the lack of information on the chemical and structural cha acteristics of the active groupings on the enzyme surface.

The most potent competitive inhibitor of carboxypeptidase previous described (1) is D-phenylalanine.[4] Significant structural elements of th compound which may be responsible for, or influence, combination with th enzyme are as follows: (1) the α-amino group, (2) the phenyl ring, (3) th α-carboxyl group, (4) the distance of separation between the phenyl rin and the carboxyl group, and (5) the optical configuration. The contrib tion of these to inhibition will now be considered, though not necessari in the order named.

The data given in Table I demonstrate that elimination of the amin group of D-phenylalanine greatly enhances the inhibitory activity of th resulting compound. Thus the affinity constant ($1/K_I$) of hydrocinnamic acid, the deaminated analogue of phenylalanine, is 32 times great than that of D-phenylalanine. Since at the pH of the measurements (p 7.5) both compounds are completely ionized, the higher affinity of hydr

[3] Snoke, J. E., and Elkins-Kaufman, E., to be published.

[4] The fact that K_m (carboxypeptidase-CGP) is 17 times greater than K_I (carbox peptidase-D-phenylalanine) does not necessarily imply that the affinity of the inhib tor for the enzyme is greater than that of the substrate, as has been previous suggested (1). Since K_m is $(k_2 + k')/k_1$, whereas K_I is k_2/k_1 (for definition of sy bols, see (1)), comparison of the relative affinities requires knowledge of either k_1 k_2 for the hydrolytic reaction, which cannot be obtained from these rate measur ments. However, K_I values for two or more specific inhibitors are in strict reciproc proportions to their respective affinities for the enzyme.

innamic acid cannot be ascribed to the lower dissociation constant of the carboxyl group. Rather, it has to be assumed that steric hindrance or lectrostatic repulsion by the amino group interferes with a close approach of D-phenylalanine to the enzyme surface.

There is strong evidence that both the phenyl ring and the carboxyl group are foci of enzyme-inhibitor interaction. If the distance of separation between these groups is increased or decreased, the resulting compounds are ess effective inhibitors than is hydrocinnamic acid. Thus the interposition of one methylene group (β-phenylbutyric acid) decreases the affinity constant 18-fold as compared to hydrocinnamic acid, whereas shortening of the chain by one methylene group (phenylacetic acid) decreases the affinity constant 6-fold (Table I). If the phenyl ring is adjacent to the carboxyl group (benzoic acid), non-competitive inhibition of relatively low order results.

If the phenyl group of hydrocinnamic acid is replaced by a methyl group (butyric acid), non-specific inhibition of undeterminate type ensues (Fig. 2). In concentrations in which hydrocinnamic acid causes strong competitive inhibition, butyric acid produces nearly non-competitive inhibition. In higher inhibitor concentrations, however, a tendency toward additional competitive inhibition is evidenced by the increase in slope at a higher ordinate intercept. A similar trend may be observed for the less active chloroacetic acid. Thus the binding of the first increments of these fatty acids by the enzyme appears to involve catalytically inactive groupings and to follow a more general pattern previously observed for the interaction of proteins with fatty acids and synthetic detergents (*cf.* (14–18)). Additional increments of bound fatty acids affect the enzymatically active groupings as well, either by direct interaction or by steric interference with the approach of substrate molecules to the enzyme surface.

The 6.4-fold decrease in affinity which results from *p* substitution of a nitro group into the phenyl ring of phenylacetic acid provides additional evidence for the contribution of the phenyl group to interaction with the enzyme. Both steric and electrostatic effects could account, however, for the lower affinity of *p*-nitrophenylacetic acid for the enzyme as compared to the parent unsubstituted acid.

A specific interaction between the enzyme and the carboxyl group of inhibitors is suggested by the well established fact that only peptides which contain this group are specific substrates for carboxypeptidase (3–5). The observed lack of inhibition by β-phenylethylamine, the decarboxylated analogue of phenylalanine, agrees with these considerations.[5]

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[5] Non-enzymatic determinations of the affinity constants of specific inhibitors for carboxypeptidase, by equilibrium dialysis measurements, are being carried out and will be reported later.

Substitution of one or two methyl groups into the amino group of
phenylalanine completely eliminates the inhibitory activity of the par
compound. This effect cannot be ascribed to intramolecular electrost
factors, since the pK of the amino group is only little increased by
substitution (compare pK_2 of glycine and sarcosine (19)). Alternativ
it has to be assumed that steric hindrance caused by the introduction
even one methyl group (N-methyl-DL-phenylalanine) is sufficient to int
fere with the combination of the amino group of D-phenylalanine with
enzyme. For the same reason, D-CGP fails to be bound by carboxypep
dase, as evidenced by its inactivity as either inhibitor or substrate.

The fact that the L isomer of CGP is hydrolyzed by carboxypeptid
suggests that the peptide group is oriented with respect to the enzy
surface in opposite direction to that of the same group in the D pepti
and opposite also to the substituted amino group of N-methyl-D-phen
alanine and N,N-dimethyl-D-phenylalanine for which it has already be
concluded that steric effects prevent attachment. However, since t
orientation of the amino group of L-phenylalanine is presumably in t
same direction as the peptide group of L-CGP, the lack of inhibition
L-phenylalanine has to be accounted for.

Kinetic measurements (1) indicate that at pH 7.5 the rate of desorpti
of L-phenylalanine is not a determining step in the kinetics of hydrolysis
L-CGP by carboxypeptidase, since L-phenylalanine, even when prese
in stoichiometric excess, does not retard the rate of hydrolysis. Accor
ingly, it has to be concluded that the positive charge on L-phenylalani
created by hydrolysis of the peptide, is sufficiently close to the acti
centers on the enzyme surface to interfere by electrostatic repulsion wi
combination. Conversely, we conclude that the positively charged ami
group in D-phenylalanine would be sufficiently distant from the enzy
surface to prevent exertion of its full electrostatic repulsion, but clo
enough for steric hindrance to be introduced by the substitution of one
two methyl groups. On the basis of this interpretation, L-phenylalani
would be expected to cause inhibition if the amino group is in the unioniz
form.[6] This has, indeed, been verified in experiments at pH 9 in whi
about 42 per cent of phenylalanine is in the *anionic* form ($pK_2 = 9.13$ (19
In these measurements, 0.04 M borate buffer, pH 9, containing 0.1 M Li
was used.[7] The substrate concentration (L-CGP) was varied from 0.
M to 0.019 M, and the concentration of L-phenylalanine maintained at
constant level of 0.0055 M (0.00233 M with respect to the *anionic* form
Strictly competitive inhibition was observed. K_m was 6.8×10^{-2}, a

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[6] This suggestion was made by Mr. S. Kaufman.

[7] Detailed measurements on the effect of pH and temperature on the hydrolysis
L-CGP by carboxypeptidase will be published elsewhere.

$_I$, calculated from the anionic concentration of L-phenylalanine in the ixture, was 5.7×10^{-3}. The corresponding affinity constant ($1/K_I$) is bout 35 per cent of that of the zwitter ionic form of D-phenylalanine at H 7.5, indicating that steric interference caused by the closer proximity f the amino group of L-phenylalanine to the enzyme surface, as compared o the D form, has a greater antagonistic effect than the electrostatic reulsion by a positive charge on the enzyme surface. The latter is probably perative in the attraction of the free carboxyl group of peptides and inibitors to the enzyme. However, steric factors are influential as well, ince complete elimination of the amino group causes an additional inrease in the affinity of the resulting compound.

The present findings that carbobenzoxyglycine and carbobenzoxyglycyllycine are entirely devoid of inhibitory activity are in accord with the bservations that these compounds are practically resistant to hydrolysis y carboxypeptidase.[8] Any appreciable degree of enzymatic hydrolysis ould require some measure of interaction with the enzyme, which should ave been detected in the present measurements in which relatively high oncentrations of these compounds were used (Table II).

In view of the recent demonstration of the hydrolysis of tryptophyl pepides by carboxypeptidase (21), the inhibitory effect of DL-tryptophan on he splitting of L-CGP was tested. Because of the greatly limited solubilty of this amino acid, only low concentrations (0.005 M with respect to -tryptophan), insufficient to cause measurable inhibition, could be emloyed.

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SUMMARY

The structural requirements of specific inhibitors for carboxypeptidase ere determined on analogues of D-phenylalanine, the most potent inhibi-

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[8] The calculated proteolytic coefficient, C, for the hydrolysis of carbobenzoxylycylglycine by carboxypeptidase (20) is only 0.02 per cent of that for the hydrolysis f L-CGP. Thus about 20,000 times the amount of carboxypeptidase would be reuired to cause the same extent of hydrolysis of carbobenzoxyglycylglycine in the ame time as is required for the hydrolysis of L-CGP. It is highly questionable hether such extremely low hydrolysis rates may be regarded as evidence for specific nzymatic activity unless it can be demonstrated that hydrolysis of the substrate is either spontaneous nor caused by the presence of traces of other enzymes in the rystalline preparation of carboxypeptidase. Similar considerations apply to the eported hydrolysis rates of carbobenzoxy-L-tryptophylglycine, carbobenzoxy-L-ryptophyl-L-proline, and carbobenzoxy-L-tryptophan by carboxypeptidase (21) hich are likewise of relatively low magnitudes.

tor previously described. Competitive inhibition of the system carb
peptidase-L-CGP was observed by the compounds hydrocinnamic a
β-phenylbutyric acid, phenylacetic acid, and *p*-nitrophenylacetic acid,
inhibitory activity of these compounds decreasing in the order named.
these, hydrocinnamic acid is 32 times more effective than D-phenylalan

From a comparison of these results with those obtained with other c
pounds, some of which were non-competitive inhibitors or inhibitors p
ducing an indeterminate type of inhibition, while others were entir
non-inhibitory, the optimal structural attributes required for spec
combination with carboxypeptidase were evaluated.

Addendum—Contrary to authoritatively expressed views (22), which have
been accepted in this paper, strictly "non-competitive" inhibition is not cha
terized by parallel lines when the data are plotted according to equation (1), si
both slope and intercept must change in order for K_m to remain constant ((23) fo
note 2). Reevaluation of the present data has shown that benzoic acid approxima
"uncompetitive" inhibition instead of "non-competitive" inhibition, whereas th
compounds which exhibit "indeterminate" inhibition fail to adhere to any of
categories described by these authors. The conclusions stated for "indetermina
and for strictly "competitive" inhibitors, therefore, remain unaltered.

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SYNTHETIC ACTION OF PHOSPHATASE*

I. EQUILIBRIA OF BIOLOGICAL ESTERS

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While several attempts have been made to calculate the free energy content of the energy-rich phosphate bonds (1–4) in intermediary carbohydrate metabolism, comparatively little is known about the energy value of the bonds of ordinary phosphate esters. We have undertaken to bridge this gap by measuring the enzymatic equilibria reached in the synthesis of various biological phosphate esters. As alcohols for phosphorylation we have used the common hexoses (glucose, fructose, mannose, and galactose), glycerol, and glyceric acid. The results with glyceric acid will be reported separately.

Although the synthetic action of unpurified phosphatase from various organs was observed at an early date with polyhydric alcohols and sugars (5–7), the enzymatic equilibrium has been measured with glycerol (7, 8) and methyl alcohol (9) only.

We have obtained the synthesis of the phosphate esters of all the natural sugars studied in the presence of alkaline phosphatase. Glucose and fructose were also phosphorylated in the presence of acid phosphatase derived from the (human) prostate gland.

Although these phosphatases seem unspecific regarding the nature of the sugar or polyhydric alcohol, the synthesized esters were all of biological configuration; *i.e.*, the hexose was phosphorylated in the 1 or 6 position or in both. Glycerol and glyceric acid were phosphorylated preponderantly on the primary alcoholic group with both alkaline and acid phosphatase.

K, the equilibrium constant, is calculated from the concentrations at equilibrium according to the equation

$$K = \frac{[\text{inorganic phosphate}] \times [\text{alcohol}]}{[\text{water}] \times [\text{ester}]} \qquad (1)$$

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in which the brackets refer to molar concentration. In determining th standard free energy (ΔF^0) from the equation

$$\Delta F^0 = -RT \ln K^0$$

the convention proposed by Lewis (10) has been followed, which assum the standard state of the solutes to be 1 molal, but that of water to be pu water and equal to 55.5 molal.[1] Therefore,

$$K^0 = K \times 55.5$$

and

$$\Delta F^0 = -RT \ln (K \times 55.5)$$

The end-points of synthesis reached for the given time intervals repr sent the true thermodynamic equilibria, at least for the main ester com ponent formed. This presupposes, of course, that the activity of th enzyme is high enough to reach such an end-point. When this is doubtfu the experiment must be discarded. In cases in which several componen esters are formed the equilibrium must be calculated for each single com pound. The accuracy of the experiments, however, permitted an exac calculation for the main component only and an estimate for the othe components present.

Materials

Phosphatase—Alkaline intestinal phosphatase purified according t Schmidt and Thannhauser (11) and a sample of acid phosphatase fro human prostate gland prepared by Dr. Schmidt[2] were used.

Sugars—The sugars used were of the purest commercial preparatio available: Merck anhydrous glucose, Merck levulose, Paragon galactos and Fisher Scientific mannose.

Glycerol—Baker's Analyzed glycerol containing 4.3 per cent moisture b weight was used.

Methods

All incubations were conducted at 38°.

Besides measuring the total amount of esterified phosphate after remov ing the inorganic phosphate by magnesia mixture, we have in every cas isolated the barium salts and analyzed them according to the usual procedures.

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[1] We thank Dr. H. Borsook for a valuable discussion of these points. The nu merical value of K in this case is the same whether molar or molal concentratio are used.

[2] We thank Dr. Gerhard Schmidt who has kindly supplied us with several batch of alkaline and acid phosphatase prepared by himself, and also privately informe us of some new modifications in the procedure of Schmidt and Thannhauser whic we have adopted to increase considerably the total yield of the purified enzym

Phosphate—Phosphate was determined by the method of Fiske and Subbarow (12) as modified by Lohmann and Jendrassik (13).

Ketose—The ketose content was determined by the method of Roe (14).

Hydrolysis—The hydrolysis curves of the barium-free esters were determined according to the procedure of Lohmann (15) at 100° and in N HCl.

Hexose Diphosphate—The amount of fructose-1,6-diphosphate present was determined with muscle zymohexase in the presence of KCN according to the procedure of Meyerhof, Ohlmeyer, and Möhle (1) and Herbert *et al.* (16).

α- and β-Glycerophosphate—For determining the amount of α-glycerophosphate in the presence of the β isomer the periodate method of Fleury and Paris (17) was used.

The initial composition of the concentrated sugar solutions was found by measuring the specific gravity, the optical rotation, and the content of inorganic phosphate. In the course of the experiments it was observed that a slow enolization took place in the sugar solutions incubated at 38° independently of any action of the enzyme, resulting in the formation of the corresponding isomers. In each experiment, therefore, the extent of the enolization was determined, *i.e.*, the aldose content in the fructose experiments and the ketose content in the aldose experiments, by measuring the increase or decrease in the Roe value of free ketose. For calculation of the equilibrium constants, these values were deducted from the concentration of the reacting sugars. Similarly, the concentrations of the corresponding phosphate esters formed therefrom were deducted from the total phosphate esters in the respective equilibria. Since the percentage of the enolized free sugars was roughly, and, in some cases, exactly, equal to the percentage of the corresponding phosphate ester, the K values were not appreciably affected.

Experimental

A representative experiment with fructose is described in detail as illustrative of the general procedure and results.

Fructose—A solution of fructose (2.7 gm.), 0.9 ml. of 2.75 M Na_2HPO_4, and 1.2 ml. of purified intestinal phosphatase (activity, 3774 units per ml. as defined by Schmidt and Thannhauser) was incubated at 38°. The concentration of organic phosphate was determined periodically. After 72 hours of incubation, when the slope of the curve obtained by plotting the organic phosphate concentration against time in hours (Fig. 1) indicated the attainment of equilibrium, the remaining reaction mixture was deproteinized with 40 per cent trichloroacetic acid. After removal of the inorganic phosphate with magnesia mixture, the barium salts were precipitated with barium acetate and alcohol, purified by reprecipitation, and dried. Weight of barium salts, 95 mg.

Analysis—22 mg. Ba salts in 14.0 ml. solution (Ba^{++} removed)

Organic P	62.7 γ P per ml.,	4.00%
Inorganic P	0.0 " " " "	0.00%
Fructose-1,6-diphosphate	2.8 " " " "	4.5% of organic P or 2.25% of hexose content

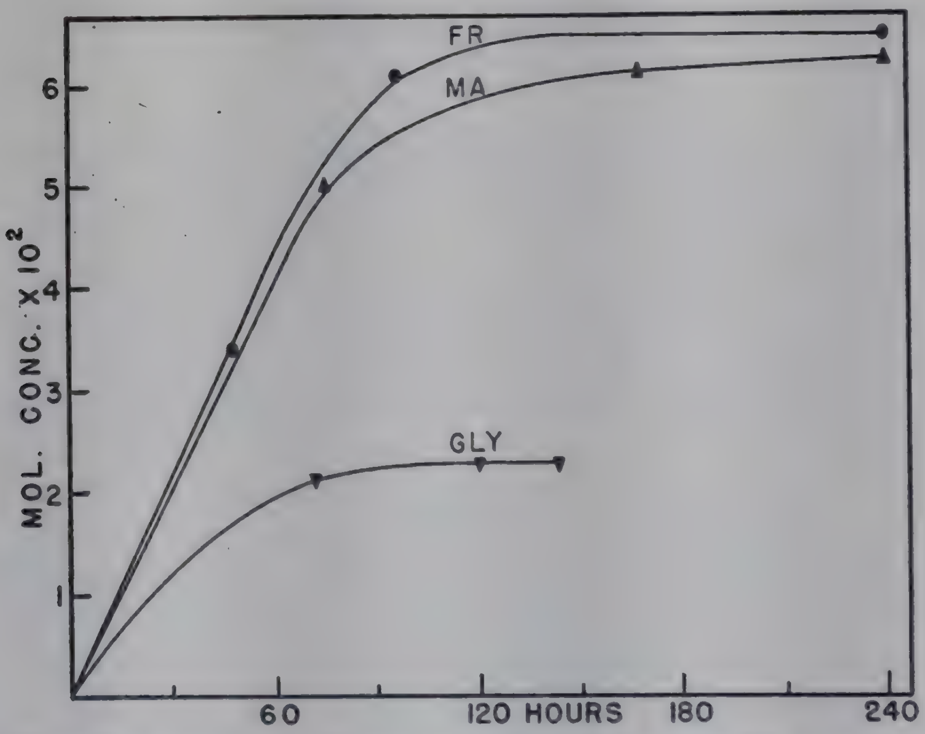

FIG. 1. Speed of synthesis. Phosphorylation of fructose (*FR*), mannose (*MA*), and glycerol (*GLY*), at 38°, pH 8.5, in the presence of purified alkaline intestinal phosphatase and Na_2HPO_4. Ordinate, molar concentration × 10^2 of phosphate ester formed. Abscissa, time of incubation in hours.

The hydrolysis values and the corresponding literature values for the component esters are given in Table I. For each time period of hydrolysis an equation of the form $ax + by + cz + dw = A$ may be written, where a, b, c, and d represent the literature values corresponding to the component esters, x, y, z, and w. A is the amount of inorganic P liberated. The solution of these equations by the method of simultaneous equations or of least squares gave the following quantitative distribution of esters: fructose-1-phosphate, 64.3 per cent; fructose-6-phosphate, 29.4 per cent; fructose-1,6-diphosphate, 2.3 per cent; glucose-6-phosphate,[3] 4.1 per cent.

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[3] The iodometric method of Willstätter and Schudel (21) as modified by Robison and MacLeod (22) was found inapplicable for the determination of the small amount of glucose-6-phosphate in the above mixture.

The main result of the fructose experiments (Tables II and III) may be briefly stated as follows: enzymatically only those esters are formed which are derived from the sugar without change in configuration. The aldose-6-phosphate may be accounted for by the phosphorylation of the aldose resulting from the partial enolization of the fructose. The high concentration of fructose-1-phosphate (65 per cent) compared to that of fructose-6-phosphate (28 per cent) indicates that the phosphate bond in the 1 position possesses much less energy than in the 6 position. Moreover, fructose-1-phosphate facilitates the phosphorylation of the 6 posi-

Table I

Distribution of Esters in Fructose Experiments

Hydrolysis at 100° in 1 N HCl			Literature values, hydrolysis			
Time	P per ml. liberated	Hydrolysis	Fructose-1-phosphate (18)	Fructose-6-phosphate (19)	Fructose-1,6-diphosphate (20)	Glucose-6-phosphate (19)
min.	γ	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
7	31.6	50.4	70.2	9.6	30.0	0.4
15	38.8	61.9	92.4	13.0	44.9	1.0
30	45.4	72.4	100.0	24.4	57.0	1.5
180	57.2	91.2	100.0	83.6	90.2	9.7

Table II

Formation of Fructose Phosphates

In all experiments but the last the pH was 8.5; in Experiment VII the pH was 5.8. The enzyme activity in all experiments but No. IVA was about 1000 units per ml.; in Experiment IVA it was 3770 units per ml. The initial water concentration in every experiment was 30.0 M.

Experiment No.	Time	Initial molar concentration		Final molar concentration						Enol	K global*
		Fructose	Phosphate	Fructose	Total ester $\times 10^2$	Fructose-1-phosphate $\times 10^2$	Fructose-6-phosphate $\times 10^2$	Fructose-1,6-diphosphate $\times 10^2$	Glucose-6-phosphate $\times 10^2$		
	hrs.									*per cent*	
M	240	4.71	0.61	(4.65)†	6.0						1.55
IV	268	4.02	0.675	3.52	6.50	4.22	2.10	0.157	0.00	11	1.24
IVA	72	4.02	0.629	3.84	6.38	4.10	1.88	0.144	0.262	3	1.17
V	552	4.02	0.655	3.48	5.88	3.82	1.34	0.241	0.458	11.9	1.34
VII	168	4.02	0.700	3.51	2.6	1.41	0.91	0.115	0.170	12	3.44

* Based upon total organic esters at equilibrium without correcting for enolization.

† Value not corrected for enolization.

tion to give 2.3 to 4.17 per cent fructose-1,6-diphosphate (and twice as much based on the phosphate esterified), as is evident from the following consideration: since the total ester represents only 1.5 per cent of the free fructose present, on the basis of random phosphorylation of the 6 position of fructose-1-phosphate (as would occur if free fructose were present in the same concentration) much less should form. The formation of about 10 times this amount of the diester shows that less energy is needed to esterify fructose-1-phosphate in the 6 position than is needed to esterify fructose itself in the same position, and even to esterify fructose in the favored 1 position. *K* for fructose-1-phosphate at pH 8.5 is 1.79; at pH 5.8, 5.5; *K* for fructose-6-phosphate at pH 8.5 is 4.2; at pH 5.8, 8.67. Finally, for fructose-1,6-diphosphate starting with fructose-1-phosphate, *K* at pH 8.5 is 0.465; at pH 5.8, 0.275; starting with fructose, *K* at pH 8.5 is 42; at pH 5.8, 69.

Table III

K Values for Fructose Esters

Experiment No.	pH	Global	Fructose-1-phosphate	Fructose-6-phosphate	Fructose-1,6-diphosphate	
					Direct	From Fructose-1-phosphate
IV	8.5	1.24	1.69	3.40	46.3	0.545
IVA	8.5	1.17	1.82	3.97	51.7	0.536
V	8.5	1.34	1.81	5.15	29.1	0.314
Average........	8.5	1.25	1.77	4.2	42	0.465
VII	5.8	3.44	5.58	8.67	69.1	0.275

Glucose, Galactose, and Mannose—The experimental conditions and procedure were essentially similar to those employed in the fructose experiment described previously. The *ketose phosphate* was determined by the method of Roe (14) and calculated as fructose-6-phosphate according to Umbreit (23).

Analysis—10 mg. Ba salt of glucose phosphate per 5.0 ml. (Ba^{++} removed).

Organic P content	90.5 γ per ml. (0.525 mg. hexose)
Roe value..............	23.5 " " " fructose
" " corrected for fructose-6-phosphate..	38.8 " " " " = 7.4% of hexose content

The hydrolysis values and the corresponding literature values for the component esters are given in Table IV. By correlating these values as described previously, the following quantitative distribution of the esters was obtained: glucose-1-phosphate 9.0 per cent, glucose-6-phosphate 83.6 per cent, fructose-6-phosphate 7.4 per cent.

Since the equilibrium ratio of glucose-6-phosphate to glucose-1-phosphate is about 20 (25), not more than 5 per cent of the latter ester can form. Because of the uncertainty of the exact distribution of glucose-1-phosphate and glucose-6-phosphate, for the purpose of calculating the equilibrium constant the amount of the latter ester was taken as the difference between the hexose content of the ester as calculated from the organic phosphorus and the ketose content as determined by the method of Roe. The same reasoning applies to the galactose and mannose phosphates for which the equilibrium ratios of the 6- to 1-esters are not known; the hydrolysis curves, however, indicate the presence of from 7 to 10 per cent of the 1-ester.

Table IV

Distribution of Esters in Glucose Experiments

Total organic P per ml. = 201 γ; hydrolysis at 100° in 1 N HCl.

Time	P per ml. liberated	hydrolysis	Literature values, hydrolysis		
			Glucose-1-phosphate (24)	Glucose-6-phosphate (19)	Fructose-6-phosphate (19)
min.	γ	*per cent*	*per cent*	*per cent*	*per cent*
7	19.0	9.45		0.4	9.6
15	25.0	12.4	100	1.0	13.0
30	31.5	15.7		1.5	24.4
90	42.0	23.3		5.2	59.6
180	61.0	30.3		9.7	83.6

Tables V and VI summarize the results of the aldose experiments. All the ketose esters herein may be considered to arise by the partial enolization of the aldose sugar and subsequent phosphorylation. If this is taken into account, then practically only aldose-6-phosphate is formed from the corresponding aldose, besides a few per cent of the corresponding aldose-1-phosphate. The average value of K for glucose-6-phosphate at pH 8.5 is 2.02; at pH 5.8 K lies between 4 and 5. The one experiment (No. VII) with a dilute solution of glucose gave a K value of 1.4. For galactose-6-phosphate at pH 8.5, K is 2.32. The one experiment with mannose gave a somewhat smaller K, 1.40.

Glycerol—The procedure of sampling and of isolating the barium salts was essentially similar to that employed in the sugar experiments. The excess NH_3 was removed, prior to the precipitation of the barium salts, by gentle boiling for a few minutes. The barium glycerophosphates were repeatedly purified by reprecipitation until the periodate determination of the α isomer gave a constant value.

An analysis of Experiment II follows: Twice purified barium salt; 10 mg. per 5.0 ml. (Ba^{++} removed); organic P = 150 γ per ml., 0.445 mg. of glycerol. A 2.0 ml. sample required 2.23 ml. of 0.00850 N I_2 for titration

TABLE V

Formation of Glucose Phosphates

The pH in all experiments but the last was 8.5; in Experiment VI the pH was 5.8. The enzyme activity was 1000 units per ml. in all experiments.

Experiment No.	Time	Initial molar concentration			Final molar concentration				Enol	K	
		Glucose	Phosphate	H_2O	Glucose	Total ester × 10^2	Glucose-6-phosphate × 10^2	Fructose-6-phosphate × 10^2		Global	Glucose-6-phosphate
	hrs.								*per cent*		
A	140	3.93	0.405	29.3	3.9	2.36	(2.12)	0.24		2.27	2.38
C	170	3.93	0.527	29.3	3.9	3.24				2.3	
IV	268	4.2	0.675	27.8	3.96	4.53	4.20	0.335	4.6	2.07	2.13
V	550	4.2	0.752	27.8	3.93	5.5	4.71	0.781	5.5	1.89	2.09
VII	216	2.38	0.555	41.5	2.27	2.16	2.04	0.082	3.8	1.4	1.43
Average....										2.00	2.02
VI	170	4.2	0.655	27.8	4.18	2.17				4.3	

Value in parentheses not corrected for enolization.

TABLE VI

Formation of Galactose and Mannose Phosphates

pH 8.5; enzyme activity, 1000 units per ml.; 9 per cent enolization.

Experiment No.	Sugar	Time	Initial molar concentration			Final molar concentration				K	
			Aldose	Phosphate	H_2O	Aldose	Total ester × 10^2	Aldose-6-phosphate × 10^2	Ketose phosphate × 10^2	Global	Aldose-6-phosphate
		hrs.									
M	Galactose	170	2.60	0.76	37	2.57	2.92			1.82	
V	"	528	2.78	0.987	37.2	2.50	3.29	2.78	0.296	2.13	2.30
VII	"	168	2.78	0.987	37.2	2.50	3.01	2.76	0.270	2.35	2.33
VI	Mannose	264	4.2	0.653	27.8	3.78	6.24	5.71	0.528	1.41	1.40

of the excess Na_3AsO_3 (0.01 N), equal to 0.438 mg. of glycerol per ml. 98 per cent α-glycerophosphate.

The procedure was repeated with thrice purified barium salt; 10 mg per 5.0 ml. (Ba^{++} removed); organic P = 164 γ per ml., 0.487 mg. o glycerol. A 2.0 ml. sample required 2.36 ml. of 0.00875 N I_2 for titratio of the excess Na_3AsO_3 (0.01 N), equal to 0.475 mg. of glycerol per ml., 97.5 per cent α-glycerophosphate.

The equilibrium for glycerophosphate has been quantitatively determined by Kay (7) with crude alkaline intestinal phosphatase and by Ohlmeyer (8) with acid phosphatase from human prostate gland. The former found $K = 0.68$ at pH 8.5 and the latter $K = 1.8$ at pH 5.8. Our own values (Table VII) are quite similar: 0.63 for alkaline phosphatase (pH 8.5) and 1.5 for acid phosphatase (pH 5.8). In one essential point, however, our results differ from those of Ohlmeyer. According to him, equal amounts of the α and β ester were formed. We found, however, that with both alkaline and acid phosphatase 98 per cent of the glycerophosphate formed consisted of the α isomer. That this comparatively high value was not due to the contamination of free glycerol resulting from insufficient separation was proved by adding glycerol to a mixture of 50 per cent α and 50 per cent β isomers and following the same procedure for the isolation and purification of the Ba salt. The same distribution of the α and β isomers was found before and after glycerol treatment. Of still greater importance was the transformation of the 50:50 mixture in the presence of phosphatase into a mixture of 95 per cent α- and 5 per cent β-glycerophosphate in 3 to 5 days.

TABLE VII

Formation of Glycerophosphate

The pH in all experiments but the last was 8.5; in Experiment 1 the pH was 5.8. The enzyme activity in all experiments was about 1000 units per ml.

Experiment No	Time	Initial molar concentration			Final molar concentration			K	
		Glycerol	Phosphate	H_2O	Glycerol	Total ester $\times 10^2$	α ester	Global	α
	hrs.						*per cent*		
2	168	7.10	0.405	27.00	6.99	11.0	98	0.69	0.71
3	144	1.7	0.480	48.8	1.677	2.26	99	0.695	0.70
4a	700	11.19	0.0863	10.3	11.14	5.52	87	0.61	0.69
4b	800	11.13	0.0863	10.3	11.13	5.83	84	0.52	0.61
Average.....								0.63	0.68
1	48	7.08	0.408	26.9	7.02	6.1	99	1.48	1.49

The great preponderance of the α isomer is not unexpected since there are two α positions for one β position; furthermore, the phosphate ester of the secondary alcohol can be assumed to have a higher energy content than that of the primary alcohol. This is best demonstrated by the analogous 3-phosphoglyceric and 2-phosphoglyceric acids which, at equilibrium, distribute in the ratio of about 4:1 (26). The high periodate value precludes the possibility that any appreciable amount of the biochemically unknown glycerodiphosphate was formed.

Finally, it seems logical that the much lower value for the *K* of the primary alcoholic phosphate ester of glycerol compared with the corresponding esters of the hexoses is also mainly due to the presence of tw equal primary alcoholic groups in every molecule. Indeed, the *K* value i

TABLE VIII

Acceleration of Phosphorylation of Glycerol

1.7 M glycerol, 0.48 M phosphate, 48 M H_2O, 0.0247 M organic phosphate added Sample, 0.5 ml.

Phosphate compound added	Glycerophosphate formed, γ P per 0.5 ml.					
	15 min.	30 min.	60 min.	120 min.	300 min.	Equilibrium (96 hrs.)
Control		16.2	19.8	32.1	77.7	266
Phosphocreatine		43.0	64.0	91.0	221.0	266
Acceleration, %		165	220	184	185	
Control	14.1	20.1	34.2			372
Phosphocreatine	42.0	74	97.0			321
Acceleration, %	200	270	185			
Control	10.8		34.2			310
Phosphocreatine	41.0		94.0			345
Acceleration, %	272		175			
Control				36.0	72.9	325
Fructose-1-phosphate				70.8	143.0	325
Acceleration, %				97	96	
Control			31.2	38.1	74.1	331
Fructose-1-phosphate			51.0	75.0	146.0	331
Acceleration, %			68	97.4	97.5	
Control			16.5	27.0	62.4	295
Glucose-1-phosphate			36.0	62.0	144.0	321
Acceleration, %			118	130	131	

a little less than half that of the most favored components of the hexos esters.

Speed of Synthesis—Fig. 1 presents some rate curves for attainment c the equilibrium in the synthesis of hexose phosphate and of glycerc phosphate. A considerably longer time is required than would be ex pected from the activity of the enzyme in the direction of hydrolysis an from the value of *K*. The latter constant, therefore, cannot be calculate from the simple formula,

$$K = \frac{k_{\text{synthesis}}}{k_{\text{hydrolysis}}}$$

A similar disparity was encountered by Bücher (27) in the study of other enzymatic phosphorylating reactions. Under our conditions the inorganic phosphate exerts a special affinity for the enzyme, thereby repressing the competitive affinity of the other reactants. The net effect is a retardation of the reaction rate not anticipated from the law of mass action.

While the speed of synthesis, consequently, cannot be theoretically explained by the activity of the enzyme and the position of equilibrium, a very interesting phenomenon was observed during the enzymatic synthesis of glycerophosphate in the presence of organic phosphate compounds of higher energy content (Table VIII). Depending upon the energy content of the added organic phosphate, the speed of synthesis of glycerophosphate was increased 100 to 300 per cent (Fig. 2). At the same time the dephosphorylation of the added organic phosphate was delayed. Whether this phenomenon is related to the transphosphorylation between aryl phosphates and certain alcohols observed by Axelrod (28) with acid citrus phosphatase is being investigated in our laboratory.

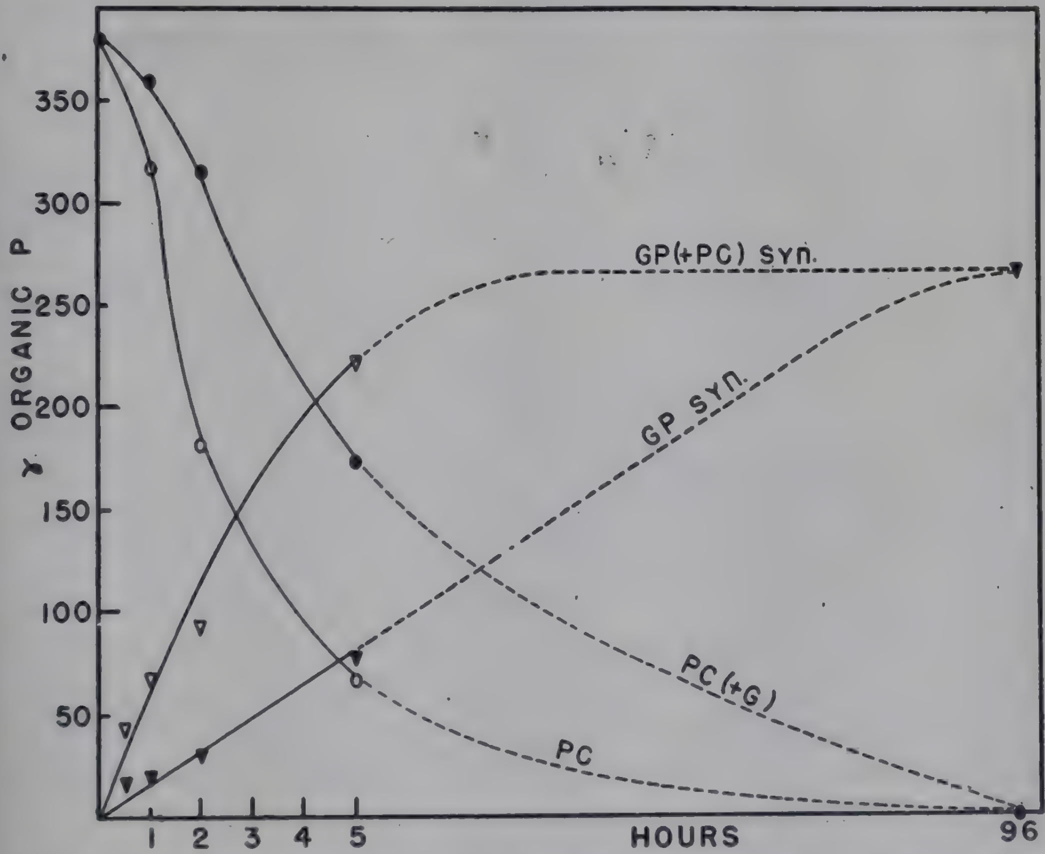

Fig. 2. Speed of enzymatic synthesis of glycerophosphate (*GP*) with and without added phosphocreatine (*PC*), at 38°, pH 8.5, in the presence of purified alkaline intestinal phosphatase and Na_2HPO_4. ▼, synthesis of glycerophosphate (control); ▽, synthesis of glycerophosphate in presence of added phosphocreatine; ○, decrease of phosphocreatine (control); ●, decrease of phosphocreatine in presence of glycerol (*G*).

Method—The amount of synthesized glycerophosphate was determined as the difference between the total organic phosphate, and of the added organic phosphate. The latter, for each time interval, determined by acid hydrolysis. A control incubation was run si taneously in order to follow the rate of hydrolysis of the added org phosphate in the absence of glycerol.

DISCUSSION

The calculation of the equilibrium constants of phosphorylatio this investigation has been based upon molar concentrations. A r exact calculation, however, involves the use of activities; *e.g.*, by ap ing the formulas of Lewis the activity coefficient of 2.38 M glucose w be 1.26. Since data are not available for all the substances and fo the concentrations, activity coefficients were not employed. Bes the use of the latter would not make the figures in the foregoing more sistent, since the tendency of the K value to become larger with increa concentration would be even more pronounced.

Although in those equilibria in which several esters are formed the termination of those present in smaller amounts is less accurate, the va obtained, nevertheless, approximate an equilibrium. This can be sh by a comparison of the K values of fructose-6-phosphate and gluco phosphate. In the presence of the enzyme phosphohexoisomerase, t two esters reach equilibrium represented by the Robison-Emden e which consists of 2 parts of glucose phosphate and 1 part of fru phosphate. Assuming that equal concentrations of glucose and fru at the same pH and temperature have the same activity, then the K v of phosphorylation of fructose-6-phosphate should be twice that of glu 6-phosphate. This is actually the case: K for the former is 4.2 an the latter 2.0 at 38°, pH 8.5 (Tables II and V).

When ΔF^0 is calculated according to formulas (2) and (3), the K 1.79 for fructose-1-phosphate and 1.4 for mannose-6-phosphate co sponds, respectively, to $\Delta F^0 = -2790$ and -2650 calories at 38°. Simil the K values of 2.2 and 2.3 for glucose-6-phosphate and galactose-6-p phate correspond to $\Delta F^0 = -3000$ calories; the K of 0.63 for gly phosphate gives $\Delta F^0 = -2200$ calories. At pH 5.8 the ΔF^0 values w be about -400 calories greater.

The discussion of the effect upon the speed of phosphorylation ca by the addition of organic phosphate of higher energy must await the sult of future experiments, in order to determine whether a transp phorylation is involved, similar to the mechanism described by Axe (28).

SUMMARY

By the synthetic action of phosphatase and subsequent isolation of esters formed, the equilibrium constant of phosphorylation was determ

for the following ordinary ester phosphate bonds: fructose-1-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, glucose-6-phosphate, mannose-6-phosphate, galactose-6-phosphate, and α-glycerophosphate. The K values for the main components of the hexose phosphates were about 2 at pH 8.5 and twice this value at pH 5.8. For α-glycerophosphate the K values were 0.63 and 1.5, respectively.

The speed of enzymatic synthesis of glycerophosphate was increased 2- to 4-fold in the presence of phosphate compounds of higher energy content. Particularly effective was phosphocreatine.

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SOME FACTORS AFFECTING THE ACETYLATION OF *p*-AMINOBENZOIC ACID IN THE RAT*

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The acetylation reaction involving detoxification of various aromatic amines both *in vitro* and *in vivo* has become of new importance with the discovery by Lipmann *et al.* (1) that the coenzyme necessary for this coupling contains pantothenic acid. In an earlier paper (2), we gave evidence demonstrating that pantothenic acid is necessary for normal acetylation of *p*-aminobenzoic acid (PAB) by rats. Although many studies have been made on the general reaction (3), the lack of agreement on results suggests that the conditions under which the measurements are made may be predominant in determining the results. It appears desirable, therefore, to reexamine some of the factors which previous work has suggested might affect the extent of the over-all coupling reaction when PAB is administered to rats. Studies reported here consist of three parts: (1) an examination of the effect of thiamine and riboflavin deficiencies on acetylation to see whether low acetylation is specific for pantothenic acid deficiency; (2) a measure of the effect of added acetate on the acetylation by deficient animals in order to eliminate low acetate supply as a possible cause of low acetylation; and (3) a measure of the effect of the amount of PAB administered on the degree of acetylation.

EXPERIMENTAL

The ability of growing rats to acetylate doses of PAB given intraperitoneally was measured by analysis of the 24 hour urine under conditions reported previously (2). All rats weighed around 100 gm. originally. Controls were fed a purified diet containing 73 per cent glucose, 18 per cent vitamin-free casein, 4 per cent corn oil (Mazola), 4 per cent Salts 4 (4), 1 per cent cod liver oil, and the following vitamins per 100 gm.: thiamine chloride 400 γ, riboflavin 800 γ, pyridoxine hydrochloride 400 γ, nicotinic acid 4000 γ, calcium pantothenate 2000 γ, and chlorine chloride 100 mg. Animals made deficient received the same diet with the appropriate vitamin omitted. In either pantothenic acid or riboflavin deficiency, the ani-

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mals survived for long periods, but in thiamine deficiency it was necessary after $2\frac{1}{2}$ weeks to supply small amounts of thiamine to the animals in order to keep them alive. They were given 15 to 50 γ of the vitamin per 100 gm. of diet thereafter and received intraperitoneal injections of 25 to 100 γ of thiamine when critical polyneuritic symptoms appeared. PAB and thiamine injections were never made at the same time. Those animals that died were replaced by others which were made deficient in the same manner as the original group.

All animals received the appropriate purified diet for 1 month before acetylation values were determined. At the end of this time, all the groups showed values which did not change appreciably over the length of the experiment for a given dose. The PAB was given intraperitoneally in single injections except in the case of the 10 mg. doses, which were given in two injections of 2 cc. each $\frac{1}{2}$ hour apart. In measuring the response to acetate, 3 or 6 per cent of the glucose in the diet was replaced by that amount of sodium acetate.

Results

Fig. 1 illustrates graphically both the effect of size of dose of PAB and of the three vitamin deficiencies upon the per cent acetylation of the PAB administered. It can be seen that low acetylation results from vitamin deficiencies other than pantothenic acid, but this is clear-cut only at the proper PAB dose level. Under each of the four nutritional conditions, the per cent acetylation of varied amounts of PAB is apparently described by two linear curves. A change in slope occurs at about the 2.5 mg. level, after which the decrease in acetylation with increasing doses is much less than at doses less than 2.5 mg. At 10 mg., all groups are approaching the same value, and none of the deficient animals shows a difference from the controls which is of statistical significance. At the lower levels (0.5 and 1 mg.), thiamine-deficient animals give values identical with the controls, and even at 2.5 and 5 mg. the difference is not striking. Values from riboflavin-deficient animals are significantly lower than from controls at all levels except at 10 mg., while values obtained during pantothenic acid deficiency are consistently below all others. The latter animals reached their minimum at 2.5 mg. and did not go below this when the dose was increased 4-fold. Such a behavior was exhibited by none of the other groups. The pantothenate curve thus appears to be distinctly different from the others, a fact which suggests that the mechanism causing lowered acetylation in this one group may be unique.

Previous work (2) on a small number of animals indicated that 3 per cent sodium acetate in the diet of pantothenic acid-deficient animals could increase acetylation significantly if a 2.5 mg. dose of PAB was given. This suggested that the acetate supply might be the limiting factor for the reaction under some conditions. Since both thiamine and riboflavin are known to function in systems involved in the formation of metabolic acetate, it appeared likely that low acetylations in these deficiencies might be due to low acetate supply. In order to examine this possibility, all three groups of deficient rats were placed on a 3 per cent sodium acetate diet and the acetylation values determined. As can be seen from Table I, in no instance did the dietary acetate have an effect. Not even when 6 per cent

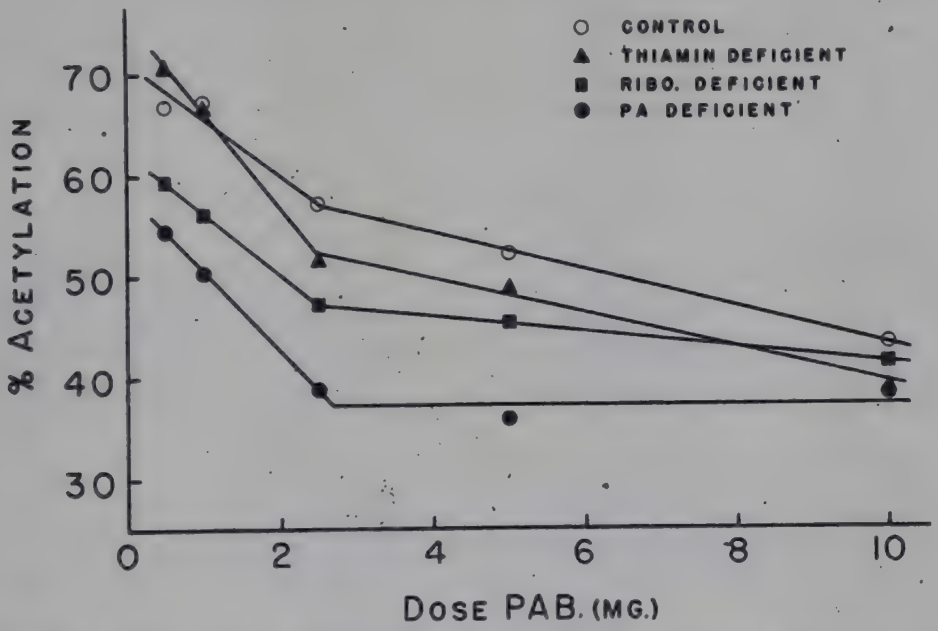

FIG. 1. The influence of nutritional deficiencies and size of dose upon the acetylation of *p*-aminobenzoic acid.

TABLE I

Effect of Dietary Sodium Acetate on Acetylation of 2.5 Mg. Doses of p-Aminobenzoic Acid by Normal and Deficient Rats

Diet	Dietary sodium acetate	No. of trials	No. of animals	Acetylation*
	per cent			*per cent*
Control		16	5	57.2 ± 1.8
Thiamine-free		17	9	51.7 ± 2.4
"	3	14	7	49.8 ± 2.9
Riboflavin-free		19	8	47.2 ± 2.3
"	3	16	8	43.7 ± 2.5
Pantothenic acid-free		21	9	38.8 ± 0.95
" "	3	18	6	35.8 ± 1.4
" "	6	12	6	43.5 ± 3.4

* Per cent bound ± the standard error of the mean.

sodium acetate was fed to the pantothenic acid-deficient group did they respond. Thus we have been unable to repeat the previous observations o pantothenic acid-deficient animals.

To examine the possibility of poor utilization of pantothenic acid by th riboflavin-deficient group, these animals were injected with either 1 or 2. mg. of calcium pantothenate simultaneously with 1 mg. of PAB. Table I shows that there was no effect.

DISCUSSION

It is apparent that a reduction in the extent that p-aminobenzoic acid i acetylated is not specific for any one nutritional deficiency. Whether th cause of the lowered acetylating ability is common in the three deficiencie is unknown, but it appears unlikely that the same defect is involved in eac case. It does seem certain that reduced food intake, while common to th three deficiencies, is not the direct cause. When the controls were fasted for a 72 hour period, and the acetylation tested during the last 24 hours of the fast, a value of 60.5 ± 2.1 per cent acetylation of a 2.5 mg. dose was obtained, compared to 57.2 ± 1.8 per cent for the same animals not fasted Furthermore, the food intake of the severely thiamine-deficient animals is practically zero, but such animals showed essentially the same per cent acetylation of a given dose as they did when only mildly deficient. The intake of food by the riboflavin- and pantothenic acid-deficient animals was usually greater than that by those deficient in thiamine, although thei ability to acetylate amine was less.

TABLE II

Effect of Injected Calcium Pantothenate on Acetylation of 1 Mg. Doses of p-Aminobenzoic Acid by Riboflavin-Deficient Rats

Diet	Calcium pantothenate injected	No. of trials	No. of animals	Acetylation*
	mg.			*per cent*
Control		10	5	67.3 ± 0.75
Riboflavin-free		22	8	56.2 ± 2.1
"	1	8	8	58.4 ± 2.7
"	2.5	8	8	59.1 ± 1.6

* Per cent bound ± the standard error of the mean.

The importance of the size of the dose of the amine to be acetylated i evaluating the possible presence of some metabolic defect is clearly show by the data. At all doses of 5 mg. or less, it is apparent that the pantothenic acid- and riboflavin-deficient animals gave results below normal but this was not evident when a low dose (0.5 or 1 mg.) was given to the thiamine-deficient group. This group showed differences from normal only in the intermediate range of the doses tested, and all of the groups approached the same value as the amount of PAB was increased to the still relatively low dose of 10 mg. The size of the dose must be considered, therefore, in attempts to compare the results of various studies upon the factors affecting the extent of acetylation of a foreign amine. Many of the data in the literature have been obtained at doses far above those used in this study. Bloomberg (5) reported that a 25 mg. dose of PAB was in the maximum range that could be completely acetylated by the human. This dosage level is of course several times less per unit of body weight than the lowest level we have tested in rats, which was 0.5 mg. to a 250 gm. animal. We have never observed individual acetylation values much above 85 per cent in any of our studies. In addition, Bloomberg gave the PAB orally, which might be expected to yield higher acetylation values owing to a less rapid rate of absorption than after intraperitoneal injection. He found a maximum of but 50 per cent absorption from the intestines, which would mean that a 25 mg. dose would amount to only a 12.5 mg. dose available for acetylation and urinary excretion. The combined data of Torda and Wolff (6) and of Bloomberg (5) show a decrease in the extent of acetylation in the human being with increasing dosage comparable to that found in this study. Also at a comparable dose per unit of body weight, the extent of the acetylation of PAB that they observed is in the same range as has been found with the rat.

The shape of the curve showing a gradual decrease in the per cent acetylated with increasing doses is undoubtedly due to several factors, which may include the rate of the acetylation reaction itself as well as the time that the test substance is in the body (*cf.* Beyer *et al.* (7)). More rapid kidney excretion of the larger doses and the different rates of elimination of the free and conjugated forms (7, 8) are certainly the major factors. It would be expected that changes in kidney function would invalidate the test as a measure of acetylation rates, but a comparison of the total amounts of the amine excreted by the animals subjected to the various deficiencies does not suggest that this is the explanation of the results obtained.

On the basis of the work of Lipmann and associates (1, 9) one can ascribe the lowered acetylation by pantothenic acid-deficient rats to a relative lack of coenzyme A, which is required for acetylation. Olson and Kaplan (10) showed that the amount of the coenzyme in various tissues falls during the development of the deficiency. According to their data, the coenzyme A content of the tissues of riboflavin-deficient animals is normal, and, as shown in the present paper, additional supplies of pantothenic acid fail to increase the acetylating ability of the riboflavin-deficient animals. It is unlikely that the amount of coenzyme A is the determini factor in riboflavin deficiency, and the same is proabably true of the thi mine-deficient animals.

Since feeding acetate in fairly large amounts failed to modify the exte of acetylation in any of the animals, it would appear that the supply acetate *per se* is not a limiting factor. Bernhard (11) and Bloch ar Rittenberg (12) have clearly shown that dietary acetate can serve in t acetylation of PAB, and the latter workers concluded that acetate is pro ably the sole precursor of the acetyl group for this compound. If this true in the deficient animals as well, then it would appear that the defect acetylation in thiamine- and riboflavin-deficient animals might be in t formation of the enzyme for acetylation, or in the activation of the aceta prior to the acetylation. Presumably a high energy phosphate bond mu be produced before the coupling can take place (13), and it is possible th interference with the metabolic pathways of carbohydrate at sever points might thus have an influence upon the extent of acetylation.

Whether differences in the degree of acetylation with the different d ficiencies can be interpreted is open to question, since one cannot measu the relative severity of the thiamine, riboflavin, and pantothenic acid d ficiencies. However, the relatively small decrease in acetylation observ in thiamine deficiency may be considered as consistent with the suggestio of Bloch and Rittenberg (12) that the major portion of the acetate aris from the oxidation of fatty acids, which does not require cocarboxylase.

SUMMARY

Acetylation studies have been made on normal rats and rats depleted thiamine, riboflavin, and pantothenic acid, with p-aminobenzoic acid the test substance.

1. In riboflavin- and pantothenic acid-deficient rats acetylation is si nificantly less than normal when 5 mg. doses of PAB or less are admini tered, the pantothenic acid-deficient animals showing consistently low values.
2. Thiamine-deficient rats show slightly less than normal acetylation 2.5 and 5 mg. PAB doses. At the levels tested above and below the amounts, the values obtained were normal.
3. When the amount of PAB was varied, all groups demonstrated a d crease in the per cent acetylated as the dosage increased. This decrea is represented graphically in each instance by linear curves which show change in slope at about 2.5 mg. of PAB. Differences in the ability acetylate the amine due to the nutritional state are evident only within limited dose range, and all animals approach the same acetylation value the maximum dose tested (10 mg. per rat). Pantothenic acid deficien

was unique in that this minimum value was obtained with a dose of 2.5 mg. per rat.

4. Supplementation of the diet of any of the deficient animals with sodium acetate had no effect upon the acetylation of a 2.5 mg. dose of PAB.

5. Excess calcium pantothenate did not alter the low acetylation values given by riboflavin-deficient rats.

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TRACER EXPERIMENTS ON THE MECHANISM OF SYNTHESIS OF VALERIC AND CAPROIC ACIDS BY CLOSTRIDIUM KLUYVERI*

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Clostridium kluyveri has been shown (1, 2) to form caproic acid from ethanol and butyric acid in accordance with the following equation:

$$CH_3CH_2OH + CH_3CH_2CH_2COOH = CH_3CH_2CH_2CH_2CH_2COOH + H_2O \quad (1)$$

This synthesis is a complex process involving numerous steps, but the net result is a condensation of a C_2 compound with a C_4 compound to give a C_6 compound. The condensation could occur theoretically in either or both of two ways. The carbinol carbon of ethanol could condense with the γ-carbon of butyric acid, or the carboxyl carbon of the acid could condense with the α-carbon of the alcohol. Several years ago (1) an attempt was made to distinguish between these two possibilities by preparing caproic acid from butyric acid labeled in its carboxyl group with C^{14}. Decarboxylation of the resulting labeled caproic acid showed that it did not contain an appreciable amount of C^{14} in its carboxyl group. This was taken to indicate that the only condensation that occurred was between the carboxyl carbon of butyric acid and the α-carbon of the ethanol. This conclusion has now been confirmed by degrading the caproic acid in such a way as to show that it is labeled only in the β position. In addition we have investigated by tracer methods the condensations that occur when valeric acid and other compounds are formed by a fermentation of ethanol and propionic acid.

Experimental Methods and Results

Degradation of Labeled Caproic Acid—The sample of labeled caproic acid prepared by the fermentation of ethanol and carboxyl-labeled butyric acid was the same as that described previously (1). It was separated from contaminating acetic and butyric acids by an azeotropic distillation procedure (3).

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The degradation of the caproic acid was done by a modification of the Barbier-Wieland procedure (4).[1] This involves the oxidative removal of one carbon atom at a time with the formation first of valeric acid and then butyric acid. Four reactions are required for the removal of each carbon atom: (1) caproic acid is converted to its methyl ester by reaction with diazomethane, (2) the ester is converted by a Grignard reaction with phenyl magnesium bromide to amyldiphenylcarbinol, (3) the carbinol is dehydrated by refluxing with acetic anhydride to form the corresponding unsaturated compound, 1,1-diphenylhexene-1, (4) the hexene is oxidized with CrO_3 in glacial acetic acid to valeric acid. The valeric acid was separated from the large excess of acetic acid by partition between ether and water and by a subsequent azeotropic distillation. The main modification, other than those required to adapt the procedure to a 0.5 mM scale, was the oxidation of the unsaturated acid on a steam bath for 10 minutes instead of at 15° for 30 minutes as recommended by Lane and Wallis (4). This increased the yield of valeric acid in the oxidation step from 13 to 73 per cent of the theoretical yield. The over-all yield in the conversion of caproic acid to valeric acid was approximately 50 per cent, and essentially the same yield was obtained in the oxidation of valeric acid to butyric acid.

TABLE I

Location of C^{14} in Caproic Acid Derived from Ethanol and Carboxyl-Labeled Butyric Acid

Carbon atom of caproic acid	Counts per min. per mM
All	1250
Carboxyl	10
α (carboxyl of valeric acid)	15
β (" " butyric ")	1300

The original caproic acid and the valeric and butyric acids obtained from the degradation were tested for purity by Duclaux distillation and were then converted to the barium salts and decarboxylated *in vacuo* at 550°. The resulting samples of barium carbonate were purified and assayed for C^{14}. The barium carbonate from the valeric and butyric acids is representative of the α- and β-carbons, respectively, of the original caproic acid.

The data given in Table I show that essentially all the C^{14} in the caproic acid was present in the β position.

Fermentation of Ethanol and Carboxyl-Labeled Propionic Acid—Clostrid-

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[1] The authors are greatly indebted to Professor W. G. Dauben for suggesting the use of this procedure.

ium kluyveri, strain K1, was inoculated into 30 ml. of a medium containing the following ingredients in gm. per 100 ml.: K_2HPO_4 0.17, KH_2PO_4 0.13, ethanol 0.58, synthetic carboxyl-labeled sodium propionate 0.66, $(NH_4)_2SO_4$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.02, $CaSO_4 \cdot 2H_2O$ 0.001, $FeSO_4 \cdot 7H_2O$ 0.0005, $MnSO_4 \cdot 4H_2O$ 0.00025, $Na_2MoO_4 \cdot 2H_2O$ 0.00025, Na_2CO_3 0.025, $Na_2S \cdot 9H_2O$ 0.02, and yeast autolysate 1 volume per cent. The culture was incubated anaerobically at 35° until fermentation ceased.

At the time of inoculation, a small aliquot of the medium was removed and analyzed for ethanol and propionic acid, and the specific activity of the latter was determined. At the end of the fermentation the products were separated, and the quantity and C^{14} content of each were estimated.

Total alcohol was estimated by the method of Widmark (5). The relative amounts of ethanol and propanol were determined by oxidizing the neutral volatile fraction with acid dichromate to the corresponding acids and determining the ratio of the latter by Duclaux distillation. No butyric acid or other higher fatty acid could be detected by partition chromatography in the products of alcohol oxidation. The fatty acids present at the end of the fermentation consisted of a mixture of acetic, propionic, *n*-butyric, *n*-valeric, *n*-caproic, and *n*-heptanoic acids. The individual acids were separated and estimated by a modification of Elsden's method of partition chromatography (6). The purity of the acids was established by Duclaux distillation. The butyric acid obtained in the first separation appeared to be slightly contaminated with labeled valeric acid and therefore was rechromatographed after addition of unlabeled valeric acid. The separated acids were converted to barium salts for the estimation of their C^{14} contents by means of a Geiger counter.

The data given in Table II show that the three products containing an odd number of carbon atoms, propanol, valeric acid, and heptanoic acid, were strongly labeled. Both the analytical data and the radioactivity measurements indicate that approximately 69 per cent of the propionic acid was converted to valeric acid, 22 per cent to propanol, and 5 per cent to heptanoic acid. The molar specific activities of the three products were approximately the same as that of the propionic acid. The somewhat lower specific activity of the heptanoic acid was probably due to contamination with octanoic acid used to pretreat the silica gel column in which the acids were separated.

The other three products containing an even number of carbon atoms, namely, acetic, butyric, and caproic acids, contained little or no C^{14}. The acetic acid showed no radioactivity. The specific activities of the butyric and caproic acid fractions were such as to indicate that not more than 1.5 and 1.2 per cent, respectively, of these acids can have been derived from propionic acid. It is possible that even the small activities observed in these fractions were due to contamination by unidentified labeled compounds.

The position of the C^{14} in the labeled valeric acid was investigated by decarboxylating a sample of the barium salt having a specific activity of 156,000 counts per minute per mM. The specific activity of the resulting barium carbonate, derived from the carboxyl group, was 162 counts per minute per mM. It can be concluded that not more than 0.10 per cent of the C^{14} was present in the carboxyl group. This is a maximum value in view of the fact that the carboxyl carbon in the barium carbonate may have been slightly contaminated with carbon from other positions in the valeric acid. Since the C^{14} was not present in the carboxyl group, it may be concluded with considerable certainty, in view of the results reported above on the degradation of caproic acid, that it was located in the β position.

TABLE II

Fermentation of CH_3CH_2CH and $CH_3CH_2C^*OOH$ by Clostridium kluyveri

Compounds		Quantity	Specific activity
		mM per 100 ml.	*counts per min. per mM*
Substrates decomposed*	Ethanol	9.14	0†
	Propionate	6.51	610,000‡
Products formed	Acetate	0.79	0
	n-Propanol	1.45	590,000
	n-Butyrate	0.75	9,200
	n-Valerate	4.50	690,000
	n-Caproate	1.50	7,500
	n-Heptanoate	0.34	530,000

* The medium initially contained 6.84 mM of propionate and 12.54 mM of ethanol per 100 ml.

† The residual ethanol at the end of the fermentation was also unlabeled.

‡ The average of the initial (620,000) and the final (600,000) values.

DISCUSSION

The degradation of C^{14}-labeled caproic acid prepared by the fermentation of carboxyl-labeled butyric acid and unlabeled ethanol has shown that the C^{14} was present almost entirely in the β position. This result proves that the caproic acid was formed by a condensation of the carboxyl carbon atom of butyric acid with one of the carbon atoms of the ethanol (equation (2)).

$$CH_3CH_2CH_2C^*OOH + CH_3CH_2OH = CH_3CH_2CH_2C^*H_2CH_2COOH + H_2O \quad (2)$$

From previous results we know that it is the methyl carbon of the ethanol that is involved in the condensation. This can be deduced from the fact hat ethanol is oxidized to acetic acid (actually acetyl phosphate (7)) and rom the fact that the carboxyl group of acetic acid is converted into the arboxyl group of caproic acid (1).

The experiment with carboxyl-labeled propionic acid and unlabeled thanol demonstrates that valeric acid is formed similarly by a condensaion of the carboxyl carbon of propionic acid with the methyl carbon of the lcohol (equation (3)). δ-Labeled heptanoic acid is probably formed from

$$CH_3CH_2C^*OOH + CH_3CH_2OH = CH_3CH_2C^*H_2CH_2COOH + H_2O \qquad (3)$$

β-labeled valeric acid and ethanol by a further condensation of the same type. The near identity of the molar specific activities of the propionic, valeric, and heptanoic acids (Table II) indicates that just one molecule of propionic acid was present in each molecule of the two higher fatty acids.

The fatty acids with an even number of carbon atoms, formed in the fermentation of carboxyl-labeled propionic acid and unlabeled ethanol, were labeled very slightly if at all. This shows that they were formed by a condensation of C_2 units derived from ethanol. The unlabeled α- and β-carbon atoms of propionic acid cannot have contributed carbon to these fatty acids, because this would involve splitting off the labeled carboxyl group of the propionic acid as carbon dioxide, and it has been shown previously (2) that carbon dioxide is not formed in appreciable amounts in this fermentation. The inability of the organism to form caproic acid from 2 molecules of propionic acid is of particular interest, since it supports the conclusion that a C_2 compound is an obligatory component of every condensation reaction that results in a lengthening of the fatty acid chain.

n-Propanol was the only alcohol formed in significant amounts in the ethanol-propionate fermentation. The close similarity in the specific activities of propanol and propionic acid indicates that the former was produced entirely by reduction of the latter compound. No comparable reduction of the higher fatty acids occurred at an appreciable rate. It is possible that some acetic acid may have been reduced to ethanol, but this would not have been detected in this experiment. In other experiments (1) the conversion of labeled acetate to ethanol was relatively slight. It therefore appears that propionic acid is the only fatty acid that is readily reduced to the corresponding alcohol under the conditions of these experiments. At present there is no obvious reason why propionic acid should be preferentially reduced.

The propionic acid-ethanol fermentation may be used as a simple and convenient method to prepare *n*-valeric acid labeled in various positions with carbon isotopes. The fermentation described in this paper gave a 69 per cent yield of β-labeled valeric acid. By fermenting unlabeled propionic acid and ethanol in the presence of methyl-labeled acetic acid,

α-labeled valeric acid can be obtained in good yield. Still other types of labeled valeric acid could be prepared by starting with α- or β-labeled propionic acid.

SUMMARY

Caproic acid formed by fermentation of carboxyl-labeled butyric acid and unlabeled ethanol with *Clostridium kluyveri* has been shown to be labeled only in the β position. This proves that caproic acid synthesis involves a condensation between the carboxyl carbon of butyric acid and a C_2 compound derived from ethanol.

By the fermentation of carboxyl-labeled propionic acid and unlabeled ethanol, it has been shown that propionic acid is converted to *n*-propanol, *n*-valeric, and *n*-heptanoic acids. The synthesis of valeric acid involves a condensation between the carboxyl carbon of propionic acid and a C_2 compound derived from ethanol. Caproic acid cannot be formed by a condensation of 2 molecules of propionic acid. The C_2, C_4, and C_6 fatty acids are derived entirely from ethanol.

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THE REDUCTION OF CYTOCHROME *c* BY XANTHINE OXIDASE

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A number of substances such as oxygen, dyes, and nitrate can act as hydrogen acceptors for xanthine oxidase (1). It has now been found that cytochrome *c* is also reduced by this system. Since only one other enzyme has been isolated which reduces cytochrome *c* (2), it was of interest to study the reaction, particularly since Ball (3) has indicated that xanthine oxidase is a flavoprotein. The activity toward cytochrome *c* was found to parallel the oxygen activity over a 200-fold range of purification, with the same substances active in the reduction of cytochrome *c* as in the reaction with oxygen. Improvement of the purification procedure has resulted in a preparation of xanthine oxidase which has a Q_{O_2} 3 times higher than Ball's best preparation, and a definite relation has been established between enzyme activity and flavin-adenine dinucleotide (FAD) content.

EXPERIMENTAL

Cytochrome *c* was prepared from beef heart according to Keilin and Hartree (4). Catalase was recrystallized three times from beef liver by the method of Sumner and Dounce (5). Hypoxanthine and xanthine solutions were 0.05 and 0.025 M, respectively, in 0.05 M NaOH, and acetaldehyde was 0.04 M in H_2O. Albumin was crystalline bovine serum albumin (Armour Laboratories).

Spectrophotometric and Manometric Tests—The reduction of cytochrome *c* was measured at 550 mμ in 1.00 cm. absorption cells in a Beckman model DU quartz spectrophotometer with a slit width of 4.6 mμ. Catalase was required to prevent the reoxidation of reduced cytochrome *c*, probably by the action of milk peroxidase which was present even in purified xanthine oxidase preparations. The test solution contained 2.48×10^{-8} mole of cytochrome *c*, 0.5 unit of catalase, 0.6 mg. of albumin, 0.04 cc. of diluted enzyme solution, and 5.00×10^{-7} mole of hypoxanthine, in 0.06 M phosphate buffer, pH 7.4, in a total volume of 1.55 cc. The gas space contained air unless otherwise indicated. The hypoxanthine solution was added last, readings were taken every minute for 7 minutes, and then about 1 mg. of solid $Na_2S_2O_4$ was added to reduce the cytochrome completely.

The concentration of oxidized cytochrome (ferricytochrome) was calculated from the equation

$$\text{Ferricytochrome} = \frac{d_R - d_t}{1.96 \times 10^4} \text{ moles per liter}$$

where d_R and d_t are the density readings after addition of $Na_2S_2O_4$ and a any time *t* during the rate determination, respectively. 1.96×10^4 is th difference between the molecular extinction coefficients for reduced an oxidized cytochrome *c* at 550 mμ. The reaction followed a first orde course until at least 80 per cent reduction, beyond which measurement were not made. A unit of enzyme was defined as the quantity which gav a value of 1.0 for (Δ log ferricytochrome)/Δ*t*, the first order rate, whe *t* was expressed in minutes. The activity, $Q_{\text{cytochrome}}$, is the number of uni per mg. of protein in the test. As shown in Table I, the rate of reacti is proportional to enzyme concentration.

TABLE I

Proportionality of Cytochrome Reduction and Oxygen Uptake to Xanthine Oxidase Concentration

Δ log ferricytochrome/Δ*t*	0.0116	0.0236	0.0491	0.0960	
Xanthine oxidase preparation, *mg.*	0.0028	0.0056	0.011	0.022	
$Q_{\text{cytochrome}}$*	4.15	4.22	4.46	4.36	
O_2 per hr., *c.m.m.*	67	143	196	250	358
Xanthine oxidase preparation, *mg.*	0.077	0.128	0.193	0.256	0.3
Q_{O_2}	870	973	1015	977	935

* Different preparations of enzyme were used for the manometric and spectroph tometric measurements.

The rate of oxygen consumption was measured manometrically at 2 essentially as described by Ball (3) except that 2.4 mg. of albumin we added to each test. Purified preparations gave little or no oxygen upta without added albumin. Philpot (6) has reported a similar observati with the methylene blue test. The albumin can be replaced by other pr teins, such as hemoglobin or cytochrome, but not by low concentratio of catalase, indicating that the protective action is not due to a remov of hydrogen peroxide.

Protein was determined by the turbidimetric procedure of Bücher (calibrated with a standardized rabbit serum solution and measured at 3 mμ with the Beckman spectrophotometer.

Purification of Enzyme—Fresh raw cream containing 40 to 42 per ce butter fat was churned in a mechanical mixer until the butter separated fine hard particles, which were removed by straining through seve yers of cheese-cloth. After addition of 0.6 volume of 0.2 M Na_2HPO_4 the buttermilk, it was digested with crude trypsin and clarified with lcium chloride as described by Ball (3). The calcium chloride superatant was warmed to 60° over a period of 5 minutes and kept there for 5 inutes, after which it was cooled in an ice bath. At this point the enzyme ses little activity in several weeks. From 2 quarts of cream about 1400 c. of calcium chloride supernatant were usually obtained.

200 cc. of the solution were treated with 45.2 gm. of ammonium sulfate nd filtered. The filtrate was treated with 12.0 gm. of ammonium sulate per 100 cc., and the precipitate was collected by centrifugation and

TABLE II

Comparison of Rate of Oxygen Consumption and Cytochrome c Reduction during Purification of Xanthine Oxidase

Enzyme preparation	Total units of cytochrome activity	Over-all yield	$Q_{\text{cytochrome}}$	$Q_{O_2}^{25°}$	$\frac{Q_{O_2}}{Q_{\text{cytochrome}}}$
		per cent			
uttermilk	1300		0.031	4.9	158
fter trypsin and heating	800	96	0.35	62	177
mmonium sulfate fraction	624	48	0.81	145	179
lumina Cγ eluate	400	31	3.50	545	156
a$_3$(PO$_4$)$_2$ "	223	17	4.96	742	150
" " *			6.78	1070	158

* Purified from a more active buttermilk preparation.

issolved in 40 cc. of water. 42 cc. of this solution were treated with 5.5 mg. of aluminum hydroxide gel Cγ (8). The centrifuged gel was luted with three 2.0 cc. portions of 0.5 M phosphate buffer, pH 7.5. The ombined eluates were treated with 9.0 cc. of saturated ammonium sulfate olution, and the precipitate was collected by centrifugation and dissolved n 10 cc. of water. The solution obtained was treated with 16.5 mg. of alcium phosphate gel (9) (aged about 3 months) and the centrifuged el washed twice with 2 cc. portions of 0.1 M phosphate buffer, pH 6.2. he enzyme was then eluted with two 2.0 cc. portions of 0.5 M phosphate uffer, pH 6.2. 6.0 cc. of saturated ammonium sulfate solution were dded to the combined eluates, and the precipitate was collected by cenrifugation and dissolved in 5 cc. of water. All operations except the wo adsorption steps were carried out at 0–2°. The final solution is stable or several days, and the enzyme may be kept in saturated ammonium ulfate for several weeks.

In Table II are shown the over-all yield and the activity with respect to oxygen consumption and cytochrome reduction at each step. The ratio of the two activities was essentially unchanged during a 220-fold purification. The reaction with xanthine was 0.6 times that with hypoxanthine in either test system. Acetaldehyde was 0.75 times as active as hypoxanthine in the spectrophotometric test; the manometric test was complicated by inactivation of the enzyme (3).

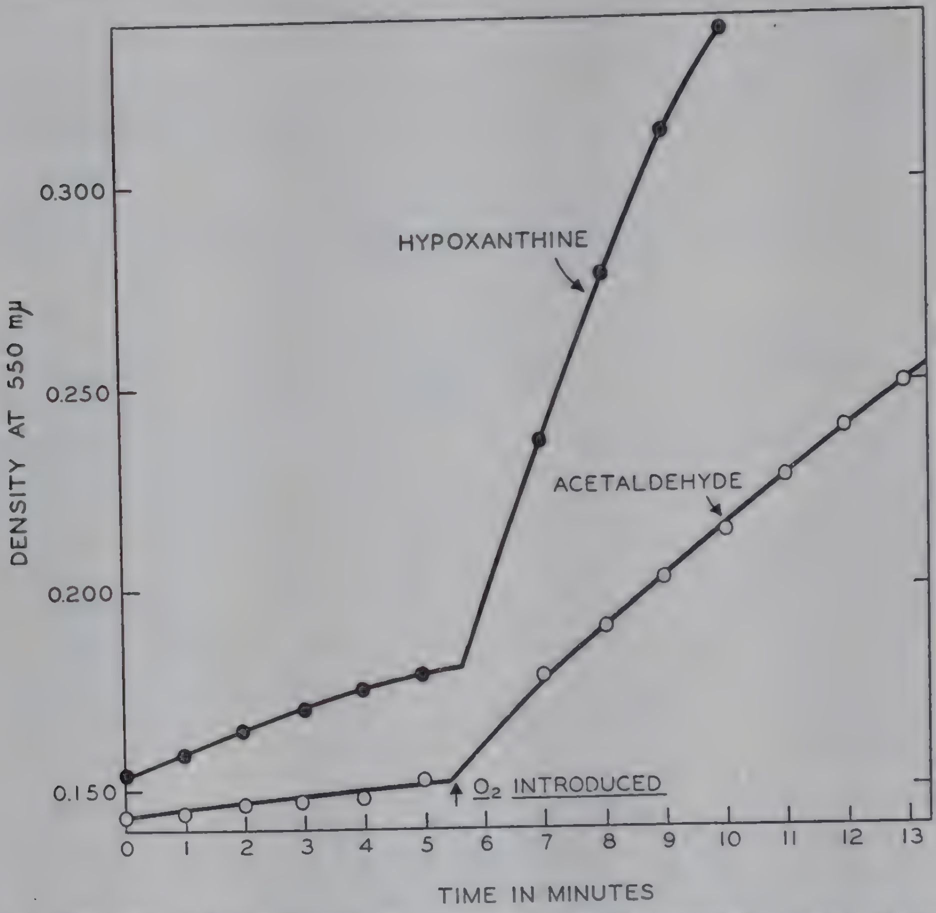

FIG. 1. The effect of oxygen on the reduction of cytochrome *c*. The first portions of the curves were obtained with solutions which had been gassed with nitrogen freed of oxygen by means of hot copper. Oxygen was bubbled through briefly at the time indicated. Test conditions were as described in the text.

The oxygen uptake at 20° was 0.75 and 0.4 of that observed at 25° and 37°, respectively. Calculation of the $Q_{O_2}^{20°}$ of the last preparation in Table II gives a value of 800, compared with a $Q_{O_2}^{20°}$ of 270 reported by Ball.

Effect of Oxygen on Rate of Cytochrome c Reduction—Oxygen was essential for the reduction of cytochrome *c*, which was very slow under anaerobic conditions. As shown in Fig. 1, this was true with acetaldehyde as well as with hypoxanthine, indicating that cytochrome *c* reduction was not due to an oxidation product of the substrate. Further, prior incubation in air with substrate before addition of cytochrome *c* did not increase the rate of reaction in the absence of air. In Fig. 2 are compared the effects of oxygen tension on the rates of reaction with oxygen and cytochrome. The reduction of cytochrome *c* continued to be accelerated up to 100 per cent oxygen. In confirmation of earlier work (10), nitrate was found to act as a hydrogen acceptor but here the reduction was inhibited by oxygen. Nitrate was reduced very much more slowly than cytochrome *c*.

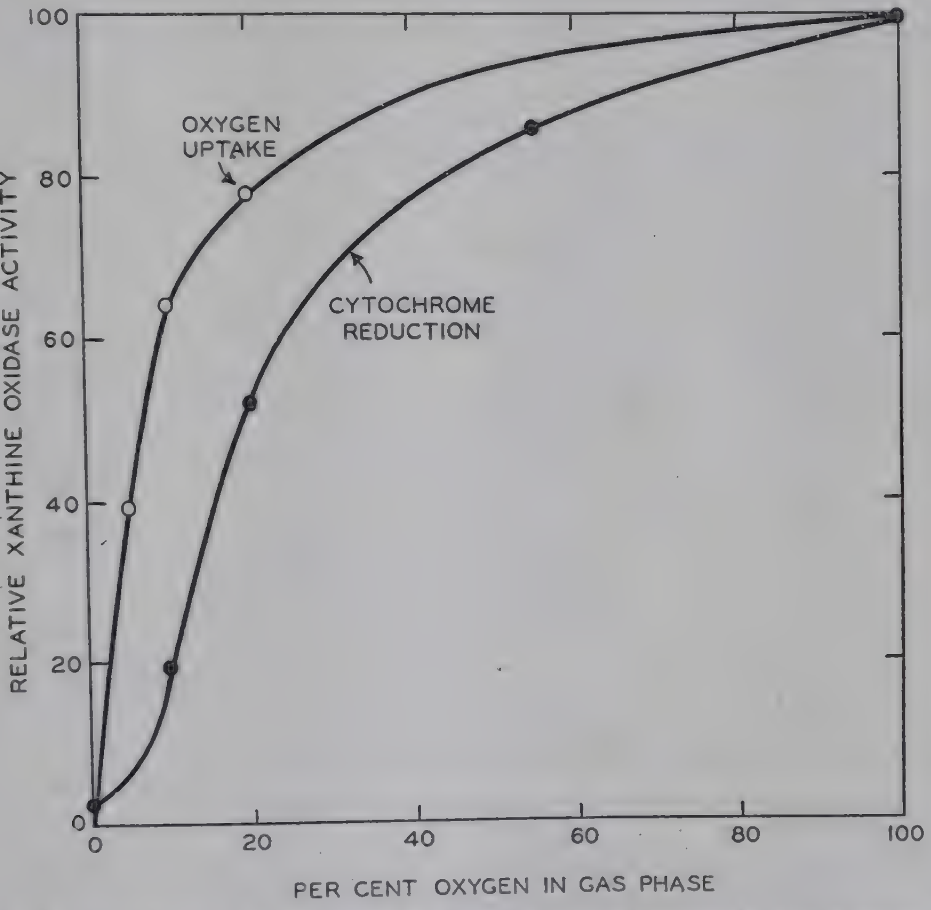

FIG. 2. A comparison of the oxygen effect in the manometric and spectrophotometric tests. Test conditions were as described in the text.

Absorption Spectrum—While the shape of the absorption spectrum of the purified enzyme was not significantly different from that reported by Ball, the absorption coefficient, β_{450}, was 2.5 times as high (3.7×10^3 sq. cm. per gm. of N *versus* 1.5×10^3). Reduction with excess hypoxanthine in the absence of O_2 resulted in partial discharge of the color; with hydrosulfite it was almost completely bleached.

From the decrease in density on reduction with hypoxanthine, the flavoprotein content of the enzyme could be calculated, assuming that the

extinction coefficient ε was 1.04 × 10^3 liters × cm.^{-1} × mole^{-1} (11, 1
and that the reduced form did not absorb at this wave-length. The flav
protein content for the preparation in Fig. 3 was 0.079 × 10^{-4} M. Wi
the further assumption that the molecular weight is 70,000 (13), the e
zyme concentration would be 0.55 mg. per cc. The purity would ther
fore be (0.55/0.89) = 0.62 (see Fig. 3).

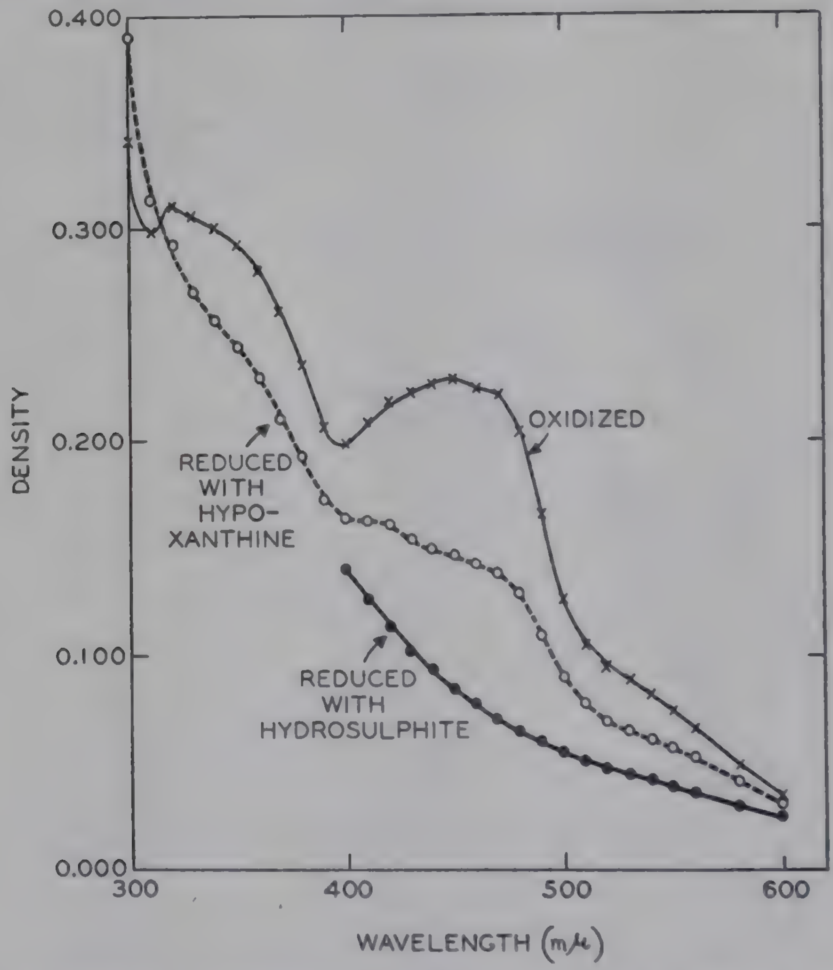

FIG. 3. The spectrum of xanthine oxidase $Q_{\text{cytochrome}} = 6.80$, $Q_{O_2} = 1070$. Each
of solution contained 0.89 mg. of xanthine oxidase, 0.39 mg. of albumin, and 3 units
catalase in 0.033 M phosphate buffer at pH 7.5. After the air was displaced by nitr
gen, hypoxanthine was added at a final concentration of 6.5 × 10^{-4} M. $Na_2S_2O_4$ w
added as the solid.

FAD Content—With a fluorometric method developed in this labor
tory[1] a direct relation was established between the enzyme activity a
the FAD content. FAD was liberated from the protein by heating
a boiling water bath for 5 minutes and the precipitated protein remov

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[1] FAD was determined by an unpublished method of A. Kornberg, based on the r
of increase in fluorescence when the dinucleotide was converted to alloxazine mo
nucleotide by the action of potato nucleotide pyrophosphatase (14).

by centrifugation. In Table III are given the results obtained with the preparations described in Table II. It is evident that the enzymatic activity and FAD content are proportional over a purification of 18-fold, in confirmation of the previous findings of Corran *et al.* (15). The last solution listed in Table III had a flavoprotein content calculated from the change in density with excess hypoxanthine of 12.1×10^{-3} micromole per cc., which is in agreement with the FAD content of 10.2×10^{-3} micromole per cc. from the fluorometric method. The residual absorption after reduction by hypoxanthine is therefore not due to a FAD-containing enzyme, since all of the FAD is present as xanthine oxidase flavoprotein.

TABLE III

Flavin-Adenine Dinucleotide Content of Xanthine Oxidase Preparations

Preparation	$Q_{\text{cytochrome}}$	Cytochrome activity	Flavin-adenine dinucleotide	Cytochrome activity / FAD
	units per mg.	*units per cc.*	*μM per cc. $\times 10^3$*	*units per μM*
After trypsin and heating	0.35	1.67	1.8	940
Ammonium sulfate fraction	0.81	2.33	2.9	810
Alumina Cγ eluate	3.50	5.72	5.0	1140
$Ca_3(PO_4)_2$ "	4.96	5.40	7.2	750
" " *	6.45	9.32	10.2	910

*From a different lot of buttermilk.

DISCUSSION

For the reduction of cytochrome *c* the turnover number in 20 per cent oxygen is 37 moles per mole of flavoprotein per minute, compared to a turnover number of 85 moles per mole of flavoprotein per minute in the reaction with oxygen. Xanthine oxidase is thus far less active than other flavoproteins in its reaction with oxygen (16) or cytochrome *c* (2). The effect of oxygen on the reduction of cytochrome requires elucidation before the rôle of cytochrome in the physiological mechanism can be understood. It would appear that the reaction with cytochrome proceeds rapidly only in the second step of the leucoflavoprotein oxidation and that oxygen is required to generate the intermediate oxidation product. Some evidence is available in support of this hypothesis, since the initial rate of reduction of cytochrome with equivalent amounts of leucoflavoprotein is very slow in the absence of oxygen, but increases as the reaction progresses.

Ball identified FAD as a constituent of his xanthine oxidase preparations, although FAD did not replace the heat-stable factor which reactivated the enzyme after prolonged dialysis. It has now been shown that

FAD quantitatively accounts for the decrease in absorption at 450 m when the enzyme is reduced with hypoxanthine. On the basis of th decrease in absorption the purity of the xanthine oxidase calculated flavoprotein is 62 per cent. It should be pointed out that with the be preparations obtained in the course of the present work the absorption 450 mμ was decreased by hypoxanthine only to the extent of 36 per cen whereas Ball obtained a decrease of 62 per cent with a preparation wi values of Q_{O_2} and β_{450} only one-fourth to one-third as high. Corran *et* (15), with a highly purified milk xanthine oxidase preparation, also foun the flavin to account for only 35 per cent of the absorption at 450 mμ.

SUMMARY

1. Xanthine oxidase purified from milk reduces cytochrome *c* in th presence of hypoxanthine, xanthine, or acetaldehyde. The activity towar cytochrome *c* parallels that toward oxygen over a 220-fold purification.

2. The rate of reduction of cytochrome *c* is accelerated by oxygen. 20 per cent oxygen the reaction with cytochrome is about one-half as fa as the reaction with oxygen.

3. An improved method of purification results in a xanthine oxida preparation which is 3 times as active as any previously reported.

4. The flavin-adenine dinucleotide (FAD) content is proportional to th enzymatic activity over a wide range in purification. The purity ca culated from the FAD content is 62 per cent. FAD accounts quant tatively for the decrease in absorption at 450 mμ when the enzyme is re duced with hypoxanthine.

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THE RELATIVE ABSORPTION OF INTRAVENOUSLY ADMINISTERED AMINO ACIDS BY THE LIVER, KIDNEY, AND MUSCLE OF THE RAT*

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The rate at which amino acids are removed from the blood by the organs has been previously reported (1–3). The methods used permitted only estimation of total amino acid nitrogen, and no information could be gained as to the fate of the individual amino acids injected.

Quantitative paper chromatography (4) seemed to lend itself as a valuable method in studying the pattern of absorption of parenterally administered amino acids. For this work, aspartic acid, glutamic acid, glycine, and alanine were chosen.

EXPERIMENTAL

Amino acid solutions were made in physiological saline and the pH adjusted to 7.4 with sodium bicarbonate. Doses equal to those given by Friedberg and Greenberg (3) were used, *i.e.* 0.16 gm. of amino nitrogen per kilo of body weight.

Male rats of the Sprague-Dawley strain, weighing 200 to 300 gm., were fasted 24 hours prior to the test. Under light ether anesthesia the solution of amino acid was injected into the lateral marginal vein on the dorsum of the hind foot. It was found quite necessary to inject slowly, consuming approximately 1 minute per ml. of amino acid solution. Circulation of the amino acid was allowed for 15 minutes and the animal was then sacrificed with nembutal given intraperitoneally. The animal was exsanguinated by severing the inferior vena cava just cranial to the liver. The liver and kidneys were removed and weighed in their entirety and weighed samples of approximately 0.5 gm. were taken for analysis. A similar sample of the gastrocnemius of the uninjected leg was also taken.

Amino acid determinations were made by paper chromatography (4). Aspartic acid, glutamic acid, glycine, and alanine were measured in each case.

RESULTS AND DISCUSSION

The data obtained are summarized in Table I as the arithmetic mean with the corresponding standard errors. Each mean is an average of the

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* This work was supported in part by a grant (No. INSTR 23) from the American Cancer Society.

results obtained from four rats, and the standard error of each mean corrected for small numbers. Differences between means were consider as significant if they were larger than 3.0 standard errors of the differen of two means.

Examination of the data shows that, of the organs studied, the kidn is the most active in concentrating the intravenously administered amin acids. Muscle tissue has the least ability in this respect, and liver is i termediate between them.

TABLE I

*Concentration of Individual Amino Acids in Organs 15 Minutes after Injection Some Amino Acids**

	Amino acid injected	Amino acids found per 100 gm. fresh tissue			
		Aspartic acid	Glutamic acid	Glycine	Alanine
		mg.	*mg.*	*mg.*	*mg.*
Liver	Aspartic acid	94 ± 11	87 ± 5	40 ± 7	45 ± 1
	Glutamic "	63 ± 7	120 ± 13	54 ± 9	55 ± 8
	Glycine	60 ± 7	80 ± 11	160 ± 48	53 ± 3
	Alanine	105 ± 14	67 ± 11	45 ± 6	160 ± 16
	None	47 ± 4	60 ± 6	40 ± 6	46 ± 5
Kidney	Aspartic acid	449 ± 31	185 ± 15	62 ± 3	37 ± 2
	Glutamic "	109 ± 5	379 ± 14	66 ± 11	38 ± 5
	Glycine	52 ± 6	99 ± 5	254 ± 7	38 ± 3
	Alanine	43 ± 2	91 ± 8	64 ± 8	167 ± 23
	None	40 ± 3	99 ± 4	63 ± 3	38 ± 5
Muscle	Aspartic acid	55 ± 10	53 ± 7	138 ± 9	44 ± 1
	Glutamic "	56 ± 13	58 ± 4	140 ± 16	47 ± 4
	Glycine	54 ± 17	58 ± 4	190 ± 4	47 ± 3
	Alanine	40 ± 3	40 ± 8	122 ± 14	67 ± 11
	None	38 ± 4	56 ± 2	138 ± 9	38 ± 4

* Arithmetic mean of values from four animals, plus or minus the standard erro corrected for small numbers.

Although the data in no way prove that the transamination reacti was involved, evidence of interconversion was found. When glutam acid was injected, aspartic acid was significantly increased in the kidne It was increased also in the liver, but the increase was not significan When aspartic acid was given, the glutamic acid was significantly increas in both the kidney and the liver. Of the organs studied, the liver seem to be the only one capable of converting injected alanine to aspartic aci Administration of glycine did not cause any changes on the concentr tions of the other amino acids measured.

In muscle the reaction seems to be relatively slow in all respects, sin conversions were not apparent and the tissue was only slightly effective in concentrating the injected amino acids, except glycine, which was concentrated to a greater extent than in liver. This absence of activity in muscle in regard to the transamination reaction does not agree with the results obtained by Cohen (5), who found skeletal muscle the most active in transamination of both glutamic acid and aspartic acid. A direct comparison of his values, obtained *in vitro*, with our values, obtained *in vivo*, is not possible. It would be necessary to take into consideration the ability of the organs examined to remove amino acids from the blood. As previously shown, the kidney concentrates aspartic acid and glutamic acid to a greater extent than liver or muscle. In fact, the uptake of glutamic acid by muscle was negligible.

The pattern of concentration for each organ was found to agree with the work of Friedberg and Greenberg (3), except for glycine and alanine in the liver. They found alanine to be concentrated in the liver to a greater degree than glycine. Our results show the opposite to be true. This difference could be due to different techniques used. They measured total amino nitrogen. Conversion of alanine to aspartic acid would not be detected. By measuring individual amino acids we found an increase in aspartic acid which would account for the lower alanine values obtained by us (Table I).

SUMMARY

By using quantitative paper chromatography, the ability of kidney, liver, and muscle to concentrate injected amino acids was studied. The pattern of concentration was found to be characteristic of each organ. Interconversions of amino acids, possibly by transamination, were demonstrated in some cases.

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α-KETO ACID-ACTIVATED GLUTAMINASE AND ASPARAGINASE

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(Received for publication, December 11, 1948)

When fresh, aqueous extracts of rat liver are heated for 10 minutes at 50°, their capacity to desamidate L-glutamine and L-asparagine is almost completely lost (1). When, however, pyruvate is added to such previously heated extracts, the capacity of the latter to desamidate glutamine and asparagine is not much less than that of unheated extracts to which the same amount of pyruvate had been added (1). These phenomena can be explained in either of two ways; namely, (*a*) the addition of pyruvate restores the glutaminase and asparaginase activity of the heated extract by some sort of reversal of denaturation, or (*b*) there are two glutaminases which differ and two asparaginases which differ, both in their heat stability and in their capacity to be activated by added pyruvate.

In case of glutaminase activity, the validity of the second of these alternatives has been shown by the recognition and separation of two glutaminases in aqueous homogenates of rat liver. One glutaminase is relatively heat-labile, sedimentable on centrifugation, and activated by added phosphate or arsenate, but not by pyruvate (glutaminase I), whereas the other is relatively heat-stable, non-sedimentable on centrifugation, and active only in the presence of pyruvate or other α-keto acid (glutaminase II) (2–8). Glutaminase I has been found in all animal tissues studied, whether normal or neoplastic (8, 9). Glutaminase II has been noted so far only in liver and in tumors derived therefrom (8, 10).

Although it appears probable that there are also two asparaginases in rat liver, no such clear-cut separation of the two has as yet been effected as in the case of the glutaminases. On high speed centrifugation of aqueous rat liver homogenates, all of the asparaginase activity is found in the supernatant.[1] However, for the time being, and on the basis of selective heat denaturation, we have tentatively designated the heat-labile fraction in rat liver as asparaginase I and the relatively heat-stable, pyruvate-activated fraction as asparaginase II.

The desamidation of glutamine by fresh, aqueous extracts of rat liver is very slow, for in the presence of the suboptimal quantities of phosphate and pyruvate in such extracts, neither glutaminase I nor glutaminase II is fully activated. It is therefore possible to study either glutaminase I or I on the unheated extract by adding either phosphate or pyruvate respectively. On the other hand, the desamidation of asparagine in rat live extracts is relatively fast, for asparaginase I appears to be almost completely active. In order, therefore, to study asparaginase II activity adequately, it is necessary to work with heated liver extracts in which asparaginase I activity is nearly completely destroyed.

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[1] Personal communication from Dr. M. Errera.

Since many of the previous investigations have been concerned with glutamine and pyruvate, we have extended these studies (*a*) to the effec on the desamidation of glutamine by another α-keto acid, namely α-ketoisocaproate, and (*b*) to the effect of both pyruvate and α-ketoisocaproate on the desamidation of asparagine.

EXPERIMENTAL

The tissues of over 75 male Osborne-Mendel rats, weighing 150 to 20 gm., were employed for these studies. The animals were sacrificed by decapitation, and the tissues were immediately removed and used within a half hour of preparation of the digests. Extracts were prepared by grinding the tissues with sand in a mortar, taking up the paste in the requisite amount of distilled water, and lightly centrifuging to remove sand an tissue débris.

The digests were composed of 1 cc. of extract, 1 cc. of veronal-acetate buffer at 0.014 M concentration (no NaCl present), 1 cc. of either water o amino acid amide, and 1 cc. of either water or salt solutions. When heated extracts were used, the practice was to plunge the digestion tube containing the 1 cc. of extract into a water bath at 50°, allowing the tube to stand at this temperature for 10 minutes, and then immediately chilling the tube in ice. The extract was brought back to room temperature and the other components of the digest added. At the end of the incubatio period (37°), the ammonia evolved was aerated over into sulfuric acid trap and determined by nesslerization. All data reported are corrected for th appropriate controls.

The glutamine was a gift of the American Cyanamid Company. O 1 minute's boiling with 1 N HCl, 90 to 95 per cent of the amide nitroge was hydrolyzed. The asparagine was a Merck product. The preparatio of sodium pyruvate has been described (6).

The α-ketoisocaproic acid was prepared by the acid hydrolysis of acety dehydroleucine (11) and was converted into the stable sodium salt b adding an equivalent amount of NaOH to the concentrated aqueous solution of the keto acid, followed by an excess of absolute alcohol. The precipitate which appeared was dissolved in the minimum amount of warm water and the solution treated with 6 times its volume of acetone. Th sodium salt of α-ketoisocaproic acid appeared in the form of glistening prisms containing 1 molecule of water of crystallization. The yield was nearly quantitative.

$C_6H_9O_3Na \cdot H_2O$. Calculated, C 42.4, H 6.4; found, C 42.5, H 6.4

On heating at 78° at 1 mm. of Hg pressure for 6 hours over phosphorus pentoxide, the compound lost 10.2 per cent in weight; calculated for 1 molecule of water of crystallization, 10.5 per cent.

No ammonia was evolved in digests of previously boiled extracts with glutamine or asparagine in the presence of either pyruvate or α-ketoisocaproate.

Studies on Glutaminase II in Unheated Tissue Extracts

pH-Activity Relations—The desamidation of glutamine in unheated rat liver extracts as a function of pH in the presence and absence of added α-ketoisocaproate is described in Fig. 1. The optimal pH in the absence of added keto acid is 8.0, and in the presence of added ketoisocaproate this pH shifts to about 8.8. The considerable acceleration of desamidation of the glutamine is also revealed in Fig. 1. For purposes of comparison, the pH-activity curve of glutamine desamidation in the presence of added pyruvate (1) is given. The marked difference in the pH optima of glutaminase activity in the presence of pyruvate and of ketoisocaproate, namely 7.2 and 8.8 respectively, reveals the influence of the nature of the keto acid on the reaction. Whether two different glutaminases II are involved cannot be answered at the present time.

Influence of Keto Acid Concentration—With increasing ketiosocaproate concentration the desamidation of glutamine in rat liver extracts reaches a maximum value, and thereafter declines (Fig. 2). In this respect, the effect of ketoisocaproate is similar to that of pyruvate and phenylpyruvate (5). Again, as with pyruvate, there appears to be a relation between the amount of ketoisocaproate necessary to bring the desamidation of glutamine to the maximum value and the amount of glutamine employed. Thus, the maximum for 14 μM of glutamine is noted when 46 μM of pyruvate (5) and 16 μM of ketoisocaproate are present, and with 28 μM of glutamine the maximum is reached with about 92 and 32 μM of pyruvate and ketoisocaproate, respectively. The molar ratio of keto acid to glutamine at the maximum is about 3 for pyruvate (5) and about 1 for ketoisocaproate.

The data in Fig. 2 were obtained by setting the pH of each digest carefully at 6.9, which is far from the optimal pH value. However, substantially the same results were obtained at pH 8.0, for although more glutamine was desamidated at this pH, the maximum desamidation occurred at 16 μM of α-ketoisocaproate when 14 μM of glutamine were used.

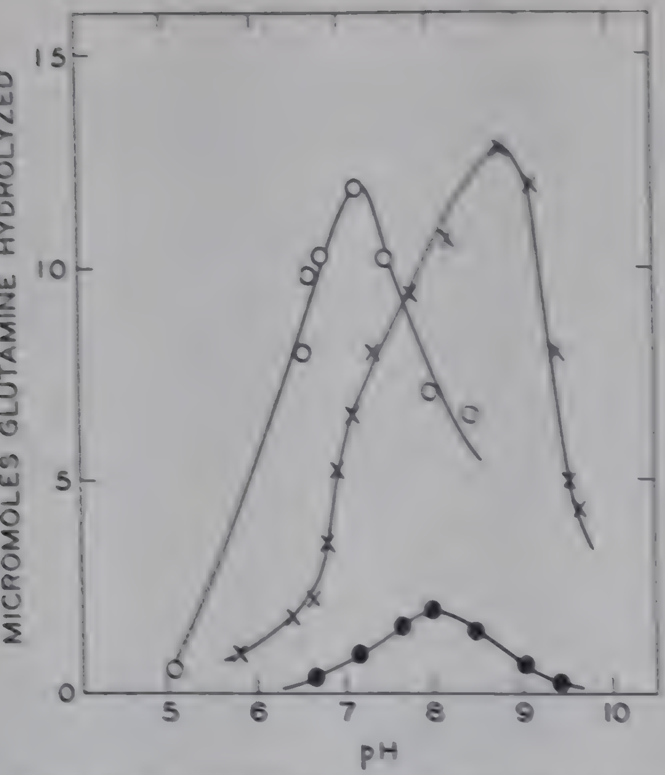

FIG. 1. Relation of pH to desamidation of glutamine in rat liver aqueous extr Veronal buffers used below pH 8.0, and glycine buffers used above pH 8.0. ● r sents glutamine desamidation in absence of added keto acid, × represents gluta desamidation in the presence of α-ketoisocaproate (16 μM), and ○ represents g mine desamidation in the presence of pyruvate (28 μM) (2). 14 μM of gluta used throughout; incubation period, 4 hours at 37°.

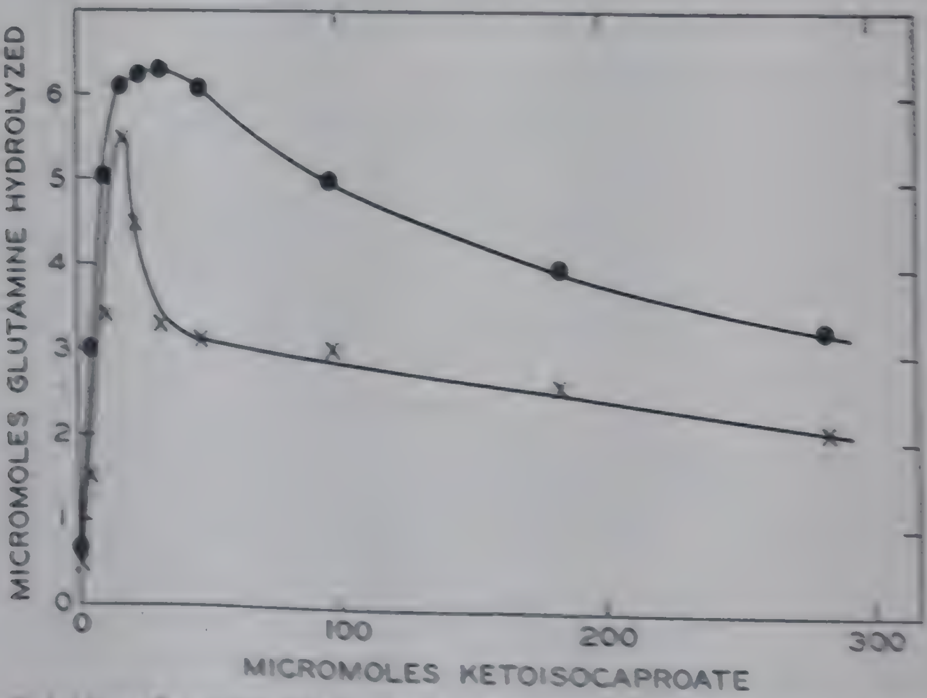

FIG. 2. Relation of concentration of α-ketoisocaproate to glutamine desamid in rat liver aqueous extracts. × represents data obtained with 14 μM of gluta and ● represents data obtained with 28 μM of glutamine. pH of each digest s 6.9; incubation period, 4 hours at 37°.

Studies with Other Rat Tissues—Aqueous extracts of rat brain, kidney, pleen, and skeletal muscle were prepared as before (6) and incubated at H 8.5 with 14 μM of glutamine in the presence and absence of 16 μM of dded α-ketoisocaproate. With these tissues, no acceleration of glutanine desamidation occurred when the keto acid was added. The findings gain show that the α-keto acid-activated glutaminase II is apparently an xclusively hepatic function (*cf.* (10)).

Studies on Asparaginase II in Heated and Unheated Tissue Extracts

pH-Activity Relations in Heated Rat Liver Extracts—Fig. 3 describes the ffect of pH on asparaginase II activity in the presence of pyruvate and -ketoisocaproate, respectively. The pH of optimal desamidation of asragine in unheated rat liver extracts is about 8.0 (1), and the same value noted for heated extracts (Fig. 3). With α-ketoisocaproate, the pH at timum activity occurs at about 7.5. There is little asparaginase activity the heated extract in the absence of added keto acid.

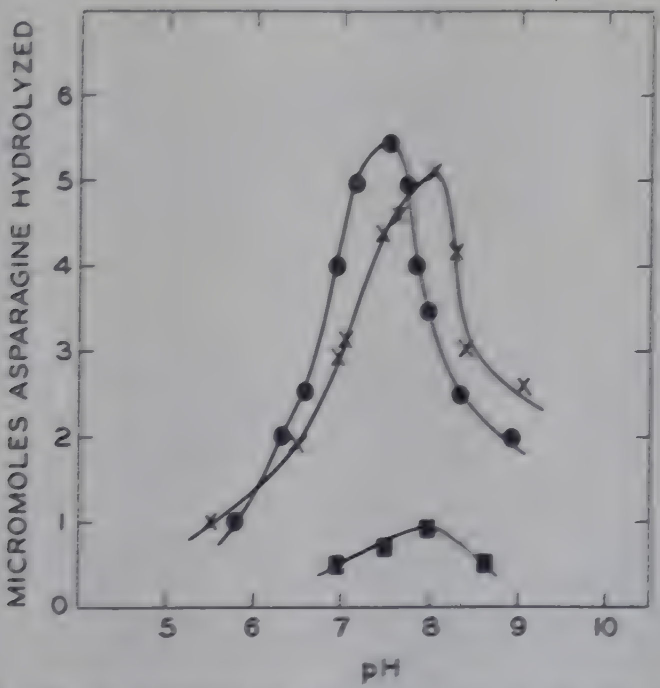

Fig. 3. Effect of pH on asparagine desamidation in heated rat liver extracts. he digests consisted of 1 cc. of heated extract equivalent to 165 mg. of tissue, 1 cc. 0.014 M veronal-acetate buffer, 1 cc. of water or 0.014 M asparagine, and 1 cc. of ater or 0.4 M sodium salt of the keto acid. ● refers to α-ketoisocaproate, × refers pyruvate, and ■ refers to digests without added keto acid. Incubation period, hour at 37°.

Effect of Added Salt Concentration on Asparaginase Activity—When soum lactate or sodium pyruvate is added to unheated rat liver extracts, ere is a relatively small but definite and reproducible increase in desamidation of asparagine (Fig. 4) (2). When added to heated extracts, sodium pyruvate produces a considerable increase in desamidation of asparagine reaching a level of activity not much less than that of the unheated extract. Sodium lactate, however, when added to the heated extract produces no activation at all. The effect of sodium α-ketoisocaproate is practically the same as that of sodium pyruvate.

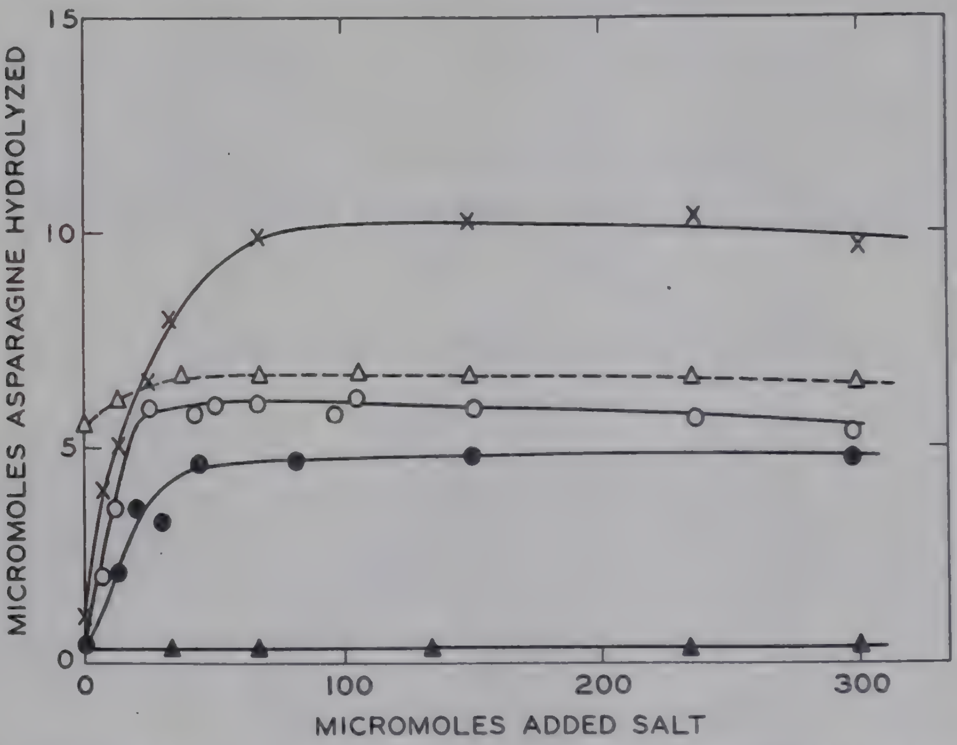

FIG. 4. Effect of added salt concentration on asparagine desamidation in rat live extracts. The continuous lines refer to data on heated extracts. The dash lin refers to data on unheated extracts. The digests consisted of 1 cc. of rat liver aqueous extract equivalent to 165 mg. of tissue, 1 cc. of 0.014 M veronal-acetate buffer a pH 8.4, 1 cc. of water or asparagine, and 1 cc. of water or salt. Final pH of the diges at every point, 8.1 ± 0.10. △ refers to sodium lactate or sodium pyruvate whic yields practically the same values with unheated extract and 0.014 M asparagine ○ refers to sodium pyruvate, ● refers to sodium α-ketoisocaproate, ▲ refers t sodium lactate in heated extracts and 0.014 M asparagine, and × refers to sodiu pyruvate in heated extracts and 0.028 M asparagine. Incubation period, 1 hour a 37°.

The curves in Fig. 4 show some interesting points of difference fro those obtained with glutamine and keto acids, and some surprising point of similarity with those obtained with glutamine and phosphate. It i recalled (*cf.* Fig. 2) (5, 7) that with increasing amounts of added α-ket acid the desamidation of glutamine reaches a maximum value and thereafter declines. With twice the concentration of glutamine (*cf.* Fig. 2 (5), about twice as much α-keto acid is needed to reach maximum desamidation, but the actual amount of glutamine desamidated is n greatly increased. On the other hand, with an increasing amount of added phosphate to either liver (12), kidney (8), or brain (6) extracts, the desamidation of glutamine reaches a maximum value and remains at this value up to relatively high concentrations of phosphate. With twice the concentration of glutamine, about twice as much phosphate is needed to reach maximum desamidation, but the actual amount of glutamine desamidated is doubled (8, 12). Reference to the curves in Fig. 4 shows that the effect of α-keto acids on asparagine desamidation in heated rat liver extracts resembles more the effect of phosphate on glutamine desamidation than it does the effect of α-keto acids on glutamine desamidation.

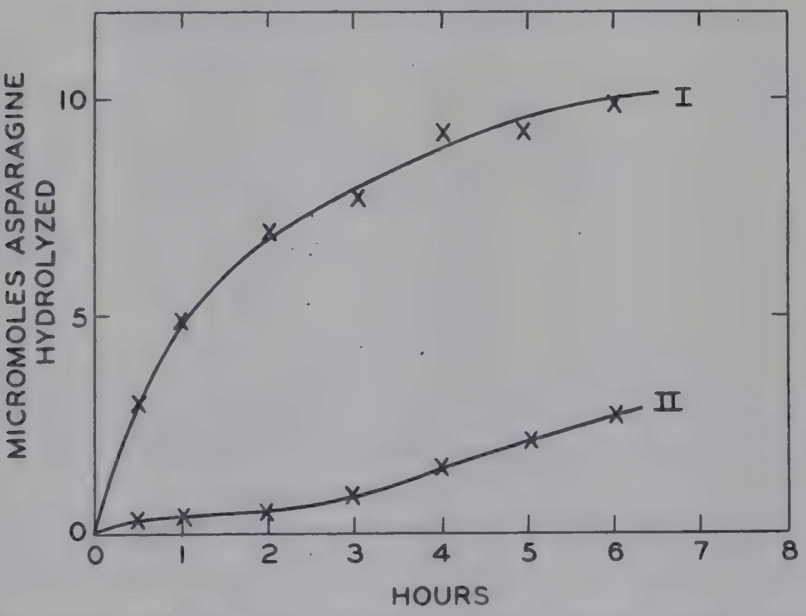

FIG. 5. Time course of the desamidation of asparagine with heated extracts of rat liver. The digests consisted of 1 cc. of extract equivalent to 165 mg. of tissue, 1 cc. of 0.014 M veronal-acetate buffer at pH 8.4, 1 cc. of either water or 0.014 M asparagine, and 1 cc. of either water or 0.023 M sodium pyruvate. Final pH of the digest at each point, 8.1 ± 0.10. Curve I refers to digests with added pyruvate, and Curve II refers to digests without added pyruvate.

The molar ratio of added α-keto acid to asparagine at maximum activation of desamidation in heated rat liver extracts is roughly 2.5 (Fig. 4).

Time Course of Desamidation of Asparagine—Fig. 5 describes the continuous time course of the desamidation of asparagine in heated rat liver extracts in the presence and absence of added pyruvate.[2]

Effect of Added Salts on Asparagine Desamidation in Heated and Unheated Extracts of Several Rat Tissues—Aqueous extracts of rat liver, kidney, spleen, and brain were divided in halves, and one-half heated as usual

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[2] Similar studies with DL-asparagine have shown that only one isomer (presumably the L form) is hydrolyzed in the presence or absence of added pyruvate.

at 50° for 10 minutes. Digests with asparagine were prepared, and th
sults of the digestion are given in Table I.

With unheated liver extracts there is the customary small but det
increase in desamidation on addition of relatively high concentratio
sodium lactate or sodium pyruvate. In the corresponding heated ext
the desamidation of asparagine almost completely disappears in the p
ence of added sodium lactate, but is almost completely retained in
presence of added sodium pyruvate.

TABLE I

Effect of Sodium Lactate and Sodium Pyruvate on Desamidation of Asparagin
*Heated and Unheated Rat Tissue Extracts**

Added salt	Micromoles asparagine hydrolyzed in extract of						
	Liver†		Kidney‡		Spleen‡		Brain
	Un-heated	Heated	Un-heated	Heated	Un-heated	Heated	Un-heated
None	4	1	8	4	3	2	1
Sodium lactate	6	1	8	4	3	2	1
" pyruvate§	6	5	8	4	3	2	1

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* The digests consisted of 1 cc. of fresh aqueous tissue extract, 1 cc. of ver
acetate buffer at pH 8.4, 1 cc. of either water or 0.014 M asparagine, and 1 cc. of e
water or 0.4 M salt solution. pH of mixture, 8.1. Extracts divided in halves b
digestion, and one-half heated at 50° for 10 minutes.

† Extract equivalent to 165 mg. of tissue per cc. Incubation period, 1 hour a

‡ Extract equivalent to 330 mg. of tissue per cc. Incubation period, 2 hou
37°.

§ Phenylpyruvate yielded the same results as pyruvate.

With unheated extracts of kidney, spleen, and brain no acceleratio
asparagine desamidation appears to occur on addition of either so
lactate or sodium pyruvate. In the corresponding heated extract,
desamidation of asparagine is uniformly diminished, whether added s
present or not. It seems reasonable to conclude that kidney, spleen,
brain do not contain asparaginase II, and that this enzyme, like glut
nase II, may be exclusively a hepatic function. Whether kidney, sp
and brain contain the same asparaginase I as liver cannot be answ
definitely at the present time. Added sodium lactate does not appar
activate asparaginase activity in these tissues as it does in liver unde
same experimental conditions (Table I).

Synthesis of β-L-Aspartyl-L-alanine—One of the explanations sugg
for the activating effect of α-keto acids on the desamidation of gluta
and asparagine is based upon the possibility that these amino acid ar
might condense with α-keto acids to form the corresponding dehydr

ides which are then hydrolyzed by active dehydropeptidases in liver to yield glutamic or aspartic acid, ammonia, and the regenerated keto acid (2, 5). One proof of this possibility would be afforded by the synthesis of such dehydropeptides as γ-glutamyldehydroalanine or β-aspartyldehydroalanine, followed by evidence that these compounds were rapidly hydrolyzed by enzymatic action in liver extracts. So far, attempts to synthesize these compounds have been fruitless. It is possible, however, to synthesize the corresponding saturated peptides, and a brief description is given of the preparation of β-L-aspartyl-L-alanine and of the failure of liver and of kidney extracts to hydrolyze it. The resistance of this saturated peptide to enzymatic hydrolysis does not mean that the corresponding analogous dehydropeptide would not be hydrolyzed under the same conditions. It is possible also that, whereas β-substituted aspartyl peptides may be enzymatically resistant, γ-substituted glutamyl peptides may be enzymatically susceptible, as shown by recent observations on the ease of enzymatic hydrolysis of glutathione in liver and kidney extracts to yield glutamic acid and cysteinylglycine (13). The problem of the keto acid activation of glutamine and asparagine desamidation is therefore still open. Methods to bring this problem to solution are being intensively explored at the present time.

Carbobenzoxy-β-L-aspartyl-L-alanine Methyl Ester-α-Benzyl Ester—17 gm. of carbobenzoxy-L-aspartyl-α-benzyl ester-β-acid chloride (14) were added in small portions and with cooling to a dry ether solution of 14 gm. of L-alanine methyl ester. The mixture was allowed to stand for a half hour and was then extracted twice with dilute HCl, once with dilute bicarbonate solution, and finally with water. After drying over Na_2SO_4, the ethereal solution was evaporated *in vacuo* to a low bulk. The product quickly crystallized on scratching, forming long needles. The yield was 9.2 gm., or 42 per cent of the theory. M.p., 102°.

$C_{23}H_{26}O_7N_2$. Calculated, C 62.5, H 5.9, N 6.4; found, C 62.4, H 5.7, N 6.5

β-L-Aspartyl-L-alanine—5 gm. of carbobenzoxy-β-L-aspartyl-L-alanine methyl ester-α-benzyl ester were dissolved in 20 cc. of acetone and treated with 15 cc. of 2 N NaOH. The mixture was shaken for ¾ hour at 25° and then treated with 25 cc. of 2 N HCl. The acetone was removed by evaporation, and the oil which appeared was taken up in ether. The ether solution was washed twice with water and evaporated *in vacuo* to a thick syrup. The syrup was not brought to crystallization but was immediately hydrogenated. For this purpose, the syrup was dissolved in a little methanol-H_2O and hydrogenated in the usual manner in the presence of palladium black (15). At the end of the reaction, the solution was evaporated *in vacuo* to a low bulk and absolute alcohol added carefully to slight excess. The peptide appeared in sheaves of prisms. The yield was 1.2 gm or 60 per cent of the theory. For analysis the compound was crystallize again from water-alcohol. M.p., 232°.

$C_7H_{12}O_5N_2$. Calculated, C 41.1, H 5.9, N 13.7; found, C 40.6, H 5.8, N 13.4

There was no evidence of water of crystallization in the compound.

Digests were prepared by mixing 1 cc. of either rat liver equivalent t 165 mg. of tissue or rat kidney equivalent to 50 mg. of tissue with 1 cc. borate buffer at pH 8.0 and 1 cc. of either water or neutralized 0.025 substrate solution. After 2 to 3 hours of incubation at 37°, the diges were examined for α-amino acid nitrogen by the manometric ninhydri method of Van Slyke. The results were completely negative. These neg tive results are consistent with those of Grassmann and Schneider, wh found that β-L-aspartylglycine was not hydrolyzed by peptidase prepar tions which acted rapidly on α-L-aspartylglycine (16).

Studies on Asparagine Derivatives—N-Carbobenzoxy-L-asparagine (1 is not desamidated when incubated with heated or unheated rat liver e tracts, whether pyruvate is added or not. It is evident that for asparagi ase activity the presence of a free α-NH_2 group in the substrate is nece sary.

The desamidation of isoglutamine in digests with rat liver extracts is n affected by added pyruvate (1–4). No effect of added pyruvate has be observed on the enzymatic desamidation of isoasparagine in digests wi heated or unheated rat liver extracts set up exactly as described above f asparagine. The relation of the α-keto acids to the desamidation of ami acid amides is apparently restricted to the ω-amides.

Recovery of Pyruvate—At the end of the incubation period, digests wi heated or unheated rat liver extracts with asparagine and pyruvate yie an 80 per cent recovery of the keto acid. This is the same as in simil digests without asparagine and shows that whatever effects pyruvate ma have on the desamidation of asparagine the keto acid is not consumed the reaction (*cf.* (1–4)).

The authors wish to acknowledge the skilful assistance of Miss Floren M. Leuthardt.

SUMMARY

1. The activation of glutaminase II by α-ketoisocaproate in digests rat liver extracts with glutamine is optimal at pH 8.8 and at a molar ra of keto acid to glutamine of about unity.

2. The separation of asparaginase I from α-keto acid-activated aspa ginase II activity in liver extracts by selective heat denaturation is

cussed, and heated preparations of rat liver extracts have been taken as sources of asparaginase II. The activation of asparaginase II by α-ketoisocaproate and by pyruvate in digests of heated rat liver extracts with asparagine is optimal at pH 7.5 and 8.0, respectively, and at a molar ratio of either keto acid to asparagine of about 2.5. With an increasing amount of added keto acid the desamidation of asparagine does not decrease, as is he case with glutamine, but is maintained at a nearly constant level. The course of the desamidation of asparagine in the presence of pyruvate s continuous with time of incubation and approaches the theoretical value.

3. Sodium lactate or sodium pyruvate added to unheated liver extracts produces a small but definite increase in the desamidation of asparagine. In heated extracts, the desamidation of asparagine in the presence or absence of sodium lactate almost completely disappears, but in the presence of sodium pyruvate it is nearly completely retained. Extracts of rat kidney, spleen, and brain do not show these activation phenomena, and it is concluded that asparaginase II is not present in these tissues.

4. The preparation of β-L-aspartyl-L-alanine is described. Neither this peptide nor N-carbobenzoxy-L-asparagine is hydrolyzed by rat tissue extracts.

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ENZYMATIC CONVERSION OF 3-HYDROXYANTHRANILIC ACID TO NICOTINIC ACID*

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The apparent conversion of tryptophan to nicotinic acid has been observed for several animal species (*cf.* (1)). Other studies demonstrated that at least some of the nicotinic acid (or amide) was synthesized within the tissues of the animal (2, 3). It was of interest to extend these studies and the ability of liver slices to synthesize nicotinic acid from its biologically active intermediate, 3-hydroxyanthranilic acid (4, 5), was investigated. The results of these studies are reported in this paper.

EXPERIMENTAL AND RESULTS

Approximately 300 mg. of liver slices (70 mg., dry weight) obtained from rats fed stock ration were incubated either in 3 ml. of Krebs-Ringer-phosphate buffer or in buffer plus 224 γ of 3-hydroxyanthranilic acid in Warburg flasks. The flasks were incubated at 38° and, after a period of 3 hours, the entire contents of each flask were treated with 10 ml. of 1 N H_2SO_4, autoclaved for 30 minutes, and the nicotinic acid content in suitable aliquots determined with *Lactobacillus arabinosus* as the test organism.

The amount of nicotinic acid present in each flask was calculated and the results expressed on the basis of 300 mg. of fresh tissue per flask. The results obtained for several experiments are shown in Table I. The values obtained for the control flasks (buffer plus tissue) were averaged within each experiment when the nicotinic acid activity attributable to additions of 3-hydroxyanthranilic acid was computed. It can readily be seen that appreciable amounts of 3-hydroxyanthranilic acid were converted to nicotinic acid in each case. The amount converted varied from 3.5 to 8.3 per cent of theoretical yield in individual tests. Liver slices were unable to convert tryptophan to nicotinic acid.

Control experiments showed that 3-hydroxyanthranilic acid was devoid of nicotinic acid activity for the test organism, *Lactobacillus arabinosus*. This is in agreement with the report of Volcani and Snell (6). In other tests, conducted as described earlier (7), 3-hydroxyanthranilic acid was also found to be inactive as a tryptophan precursor for *Lactobacillus arabinosus*.

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This organism is therefore well suited for measuring the nicotinic a active compounds in these experiments.

These *in vitro* experiments afford further proof for the tissue synth of nicotinic acid. The detailed study of the enzymatic steps and fac which influence them should afford significant information on the pathw involved in nicotinic acid synthesis from tryptophan.

TABLE I

Conversion of 3-Hydroxyanthranilic Acid to Nicotinic Acid

Experiment No.	Flask No.	Contents	Nicotinic acid produced		
			Per 300 mg. liver slices	From 3-hydroxyanthranilic acid	As cent ret yiel
			γ	γ	
1	I	Control	20.0		
	II	3-Hydroxyanthranilic acid	31.0	9.85	5.
	III	Control	20.3		
	IV	3-Hydroxyanthranilic acid	35.2	15.05	8.
2	I	Control	18.4		
	II	3-Hydroxyanthranilic acid	30.9	12.3	6.
	III	Control	18.8		
	IV	3-Hydroxyanthranilic acid	27.5	8.9	4.
3	V	Control	15.8		
	VI	3-Hydroxyanthranilic acid	25.1	9.3	5
	VII	" "	22.1	6.8	3.
4	I	Control	29.1		
	II	"	25.6		
	III	3-Hydroxyanthranilic acid	40.5	13.1	7.
	IV	" "	35.2	7.8	4.

* Molar basis.

SUMMARY

The ability of liver slices to convert 3-hydroxyanthranilic acid to tinic acid was investigated. It was found that from 3.5 to 8.3 per conversion occurred when 300 mg. of slices were incubated with 2 of 3-hydroxyanthranilic acid for a period of 3 hours.

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SYNTHESIS OF α-AMINO-β-HYDROXY-n-BUTYRIC ACIDS

VII. AN IMPROVED SYNTHESIS OF DL-ALLOTHREONINE

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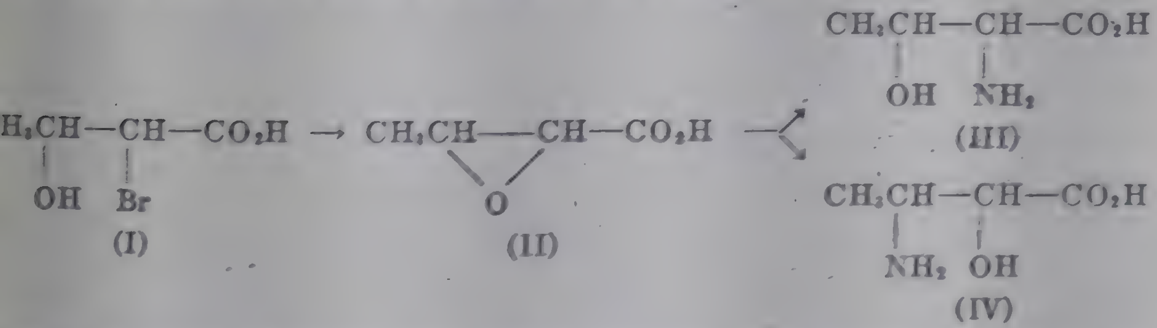
(Received for publication, December 21, 1948)

Several methods are available for the synthesis of DL-threonine (1–3). owever, each of these involves the separation of threonine from a mix-ure containing varying proportions of the isomeric allothreonine. Re-ently it has been reported that derivatives of DL-allothreonine may be onverted to DL-threonine in excellent yields (3–5). These developments ake the preparation of DL-allothreonine of interest as a key intermediate the synthesis of DL-threonine.

Although several methods for preparing allothreonine have been de-cribed (6, 7), none of these is entirely satisfactory. In considering other ynthetic routes to this amino acid, it was decided to investigate the reac-on of α-bromo-β-hydroxy-n-butyric acid with ammonia.

As has been pointed out previously (8) α-halogen-β-hydroxy acids, when eated with ammonia, are converted into glycidic acids which may add mmonia at either the α- or the β-carbon atom (9). Thus in the amina-on of α-bromo-β-hydroxy-n-butyric acid (I) there is the possibility that e epoxide intermediate (II) may yield either the α-amino-β-hydroxy acid II), the β-amino-α-hydroxy acid (IV), or a mixture of the two.



The literature indicates that either reaction may occur, depending on e particular compounds employed. Thus α-chloro-β-hydroxypropionic cid (10) and glycidic acid (11) react with ammonia to give isoserine, hereas ethyl α-bromo-β-hydroxypropionate on treatment with benzyl-nine gives N-benzylserine ethyl ester (12). α-Bromo-β-hydroxy-β-phen-propionic acid and β-phenylglycidic acid with ammonia give a mixture of phenylserine and phenylisoserine in which the latter predominates (13 whereas with aromatic amines the α-amino-β-hydroxy acid is formed (14 Melikoff (15) reported that amination of 2-methylglycidic acid yielde β-amino-α-hydroxy-*n*-butyric acid, but gave no evidence for the structur of the product. More recently Burch (16) concluded, on the basis o slight evidence, that the product obtained by amination of α-chloro-β-hydroxy-*n*-butyric acid was a mixture of the two aminohydroxy acids. I seemed desirable, therefore, to investigate this reaction further.

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* Smith, Kline and French Laboratories Walter G. Karr Fellow, 1948–49.

It was found that α-bromo-β-hydroxy-*n*-butyric acid reacts smoothl with aqueous ammonia either at room temperature or at 65–75°, givin the aminohydroxy acid in 80 to 90 per cent yield. The crude product gav N-benzoyl-DL-allothreonine in 90 per cent yield. The unrecrystallize derivative melted at 173–174° (pure N-benzoyl-DL-allothreonine melts a 176–177°). Several attempts were made to isolate other benzoyl derivatives from the mother liquors but with no success. No evidence was obtained for the presence of β-amino-α-hydroxy acids or of DL-threonine It would appear, therefore, that the reaction of α-bromo-β-hydroxy-*n* butyric acid with ammonia is highly specific, not only structurally bu also stereochemically, since only one of the four possible DL-aminohydroxy *n*-butyric acids is produced.

The excellent results in the amination step made this procedure a ver promising one, provided a satisfactory method of preparing α-bromo-β hydroxy-*n*-butyric acid could be developed. Melikoff (17) obtained thi substance by treating crotonic acid with a solution of hypobromous aci prepared from bromine and water in the presence of mercuric oxide. Onl one of the two possible racemic forms of the α-bromo-β-hydroxy acid wa obtained. Since this method did not appear practical for the preparatio of the acid in quantity, the convenient technique employed by Read an Andrews (18) in making the bromohydrin from cinnamic acid was studied By passing bromine vapor into an aqueous solution of crotonic acid, a hig yield of the crude hypobromous acid addition product was obtained, together with a small amount of the dibromo acid. The crude product wa easily purified to give α-bromo-β-hydroxy-*n*-butyric acid in 79 per cen yield. In agreement with the results of Melikoff (17) only one racemi form of the bromohydroxy acid, m.p. 86–87°, could be isolated from th reaction mixture. The highest yield was obtained by brominating a 0.3 M solution of crotonic acid; increasing the crotonic acid concentration t 0.67 M led to a decreased yield of bromohydroxy acid together with an increase in the amount of dibromo acid formed.

This procedure provides an excellent method for the synthesis of DL allothreonine, since it gives a high yield of a product which contains littl or no DL-threonine or β-amino-α-hydroxy-*n*-butyric acid.

EXPERIMENTAL

α-Bromo-β-hydroxy-n-butyric Acid—A solution of 23.2 gm. (0.27 mole) of crotonic acid in 800 ml. of water (0.34 M) was placed in a 4 liter beaker and cooled to 0–5°. The solution was stirred vigorously while a stream of air charged with bromine vapor was introduced. The bromine vapor-air mixture was obtained by bubbling a slow stream of air through 45 gm. of bromine (0.28 mole) in a gas-washing bottle at room temperature and was passed into the reaction vessel through a glass tube terminating in a perforated bulb. The air stream was regulated so that the reaction mixture remained colorless or became only slightly yellow. The addition required about 5 hours (with less vigorous stirring, 8 hours). After all the bromine was added, air was bubbled rapidly through the reaction mixture for an additional 30 minutes to remove any excess bromine. The colorless solution was decanted from a small amount of brown oil (probably crotonic acid dibromide) adhering to the sides of the beaker and was extracted with two 150 ml. portions of low boiling petroleum ether. The aqueous solution was saturated with sodium chloride and extracted with three 200 ml. portions of ether. The ether and the petroleum ether extracts were dried over magnesium sulfate and the solvents were removed *in vacuo*. The petroleum ether extract gave 2.0 gm. of crude crotonic acid dibromide which after recrystallization from benzene melted at 86–87°. (Michael and Norton (19) reported a melting point of 87° for this acid.) This material liberated iodine immediately from a solution of sodium iodide in acetone. The ether extract yielded a pale yellow oil which crystallized on standing several days in a vacuum desiccator over phosphorus pentoxide. (In subsequent runs rapid crystallization was induced by seeding with crystalline material.) The yield of crude α-bromo-β-hydroxy-*n*-butyric acid was 44.0 gm. (89 per cent based on crotonic acid). This material slowly liberated iodine from an acetone solution of sodium iodide. It was triturated with 11 ml. of a benzene-petroleum ether mixture (1:2 by volume) and washed with 6 ml. of the same solution, giving an almost white solid melting at 84–85°. A second trituration and washing with benzene-petroleum ether gave almost pure α-bromo-β-hydroxy-*n*-butyric acid in 79 per cent yield. This material melted at 85–86° and did not liberate iodine from sodium iodide in acetone. A mixture of this substance and crotonic acid dibromide melted at 62–64°. Pure α-bromo-β-hydroxy-*n*-butyric acid melting at 86.5–87° was obtained by recrystallizing the partially purified material from benzene-petroleum ether (Melikoff (17) reported a melting point of 86–87° for this acid). The partially purified product was entirely satisfactory for use in the amination step.

Increasing the concentration of the crotonic acid solution to 0.67 M lowered the yield of crude bromohydroxy acid to 81 per cent and increased that of the dibromo acid to 18 per cent. Decreasing the concentration crotonic acid below 0.34 M did not seem to improve the yield and extra tion of the bromohydrin from the more dilute solutions was less comple

DL-*Allothreonine*—(*a*) A solution of 20.0 gm. (0.109 mole) of pure bromo-β-hydroxy-*n*-butyric acid in 200 ml. of 28 per cent aqueous a monia was allowed to stand at room temperature for 4 days. The colorle reaction solution was concentrated *in vacuo*, giving a white crystalli residue. This was dissolved in a minimum of hot water and 10 volum of hot absolute alcohol were added to the solution. The mixture w allowed to cool to room temperature and then was placed in the ice b overnight. The white crystalline precipitate was filtered and washed wi absolute alcohol, then ether. The air-dried product, which contained small amount of ammonium bromide, weighed 9.0 gm. By adding a solute alcohol to the filtrate and allowing it to stand overnight, a seco crop of crystals (1.9 gm.) was obtained. The total yield of crude produ (m.p. 234–235°) was 84 per cent of the theoretical amount. Recrysta lization of this material as described above gave DL-allothreonine melti at 242–243° (88 per cent recovery). A sample of pure DL-allothreoni (1) in the same bath melted at 244–245° and did not depress the melti point when mixed with the product above. The over-all yield based the quantity of crotonic acid used was 59 per cent of the theoretical amoun

Benzoylation of 5.0 gm. (0.042 mole) of the crude product by the meth of Carter and Stevens (20) yielded 9.6 gm. of crude benzoyl derivati (m.p. 175–176°), which contained some sodium chloride. Recrystalliz tion of this material from 40 ml. of hot water gave 8.3 gm. of N-benzoy DL-allothreonine (58 per cent yield based on crotonic acid, m.p. 177°).

The N-benzoyl derivative was obtained directly from the aminati mixture by the following procedure. To the amination solution (pr pared from 10 gm. (0.055 mole) of bromohydroxy acid as described abov were added 32 ml. of 2 N sodium hydroxide. The solution was evaporat to dryness under reduced pressure to remove ammonia. The residue w dissolved in 17 ml. of water and 38 ml. of 2 N sodium hydroxide. To t solution were added in portions 13 ml. of benzoyl chloride and 130 ml. 2 N sodium hydroxide in the usual way. The reaction mixture was work up according to the procedure of Carter and Stevens (20). After recryst lization of the crude benzoylation product (m.p. 173–174°), N-benzoyl- allothreonine was obtained in 50 per cent yield based on crotonic acid.

(*b*) The amination described above was repeated with crude α-bromo hydroxy-*n*-butyric acid. Crude allothreonine was obtained in 46 per c yield calculated on the amount of crotonic acid used. The water-alco filtrate was evaporated to dryness *in vacuo*. The residue, consisti mainly of ammonium bromide, was dissolved in water, the solution ma alkaline with 2 N sodium hydroxide, and concentrated under reduced pr sure to remove ammonia. Benzoylation of the resulting solution by the method of West and Carter (21) gave an oily product from which no pure benzoyl derivative could be isolated.

When the crude α-bromo-β-hydroxy-*n*-butyric acid was allowed to stand in the ammonia solution for only 2 days, the yield of crude allothreonine was lowered to 40 per cent (based on crotonic acid).

(*c*) A solution of 43.7 gm. (0.238 mole) of pure α-bromo-β-hydroxy-*n*-butyric acid in 440 ml. of 28 per cent ammonia water was heated overnight in sealed bottles on the water bath (65–75°). The slight yellow reaction solution was filtered to remove a trace of gelatinous precipitate and worked up as described above. The once recrystallized product melted at 222–224°. Benzoylation of 5.0 gm. (0.042 mole) of this material in the usual way yielded 8.8 gm. of crude benzoyl derivative (m.p. 172–174°). An additional 0.5 gm. of solid was obtained by concentration of the filtrate *in vacuo* to one-half its original volume. After recrystallization from water both fractions gave pure N-benzoyl-DL-allothreonine (56 per cent yield based on crotonic acid). Upon standing the filtrate from the second crop of crystals deposited 0.2 gm. of solid, m.p. 136–140°, from which no pure substance was obtained after repeated crystallizations from water.

The alcohol-water filtrate from the crude amination product was evaporated to dryness under reduced pressure and benzoylated as described in (*b*). 1.7 gm. of crude product were obtained, which, after several crystallizations from water, gave 1.0 gm. of N-benzoyl-DL-allothreonine. No N-benzoyl-DL-threonine or N-benzoyl-β-amino-α-hydroxy-*n*-butyric acid could be isolated from the reaction mixture.

SUMMARY

1. Amination of α-bromo-β-hydroxy-*n*-butyric acid gives DL-allothreonine in high yield. No β-amino-α-hydroxybutyric acid was isolated from the reaction.
2. α-Bromo-β-hydroxy-*n*-butyric acid has been prepared in 79 per cent yield by bromination of crotonic acid in aqueous solution.
3. This method for preparing DL-allothreonine is a considerable improvement over previous syntheses, since it involves fewer steps, gives higher yields, and the crude product does not contain appreciable amounts of any of the other aminohydroxybutyric acids.

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THE SOURCE OF AMMONIA IN PLANT TISSUE EXTRACTS

II. THE INFLUENCE OF UREA

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In a previous paper (1) we have drawn attention to the discrepancy in ree ammonia values of fresh and dried green leaf tissue. The evidence as derived largely from the pasture grasses but the same observation as made with other species; *viz.*, that in practically all cases the ammonia f dried tissue was significantly higher than that of fresh tissue. It was hown that the increase in ammonia due to drying could not be attributed to the hydrolysis of glutamine in the oven, and we tentatively ostulated the presence in leaves of an ammonia precursor which is even ore heat-labile than glutamine.

Attempts were then made by infiltration techniques to increase the oncentration of this ammonia precursor as measured by the difference etween fresh and dried ammonia values. A wide variety of nitrogenous ompounds was introduced into the leaf and it was found that urea caused he largest increase in the concentration of ammonia precursor. The ossibility that urea was responsible for the increase in ammonia on drying was therefore investigated and in this paper are recorded certain observations on the metabolism of urea in plants.

EXPERIMENTAL

Methods for the preparation of plant extracts and analytical procedures for the estimation of ammonia and glutamine are as described in the previous paper (1). Urea was determined by means of urease, the resultant ammonia being distilled *in vacuo* and estimated by hypobromite oxidation. Jack bean meal, commercial samples of urease, and crystalline urease were used at various times throughout the investigation.

Urea determinations have been made on some hundreds of samples of grasses under a wide variety of environmental conditions and on a limited range of other plants, including peas, beans, silver beet, clovers, tomatoes, etc., under ordinary field conditions. Values between 1 and 5 mg. of urea N per 100 gm. of fresh tissue were usually obtained, the values being almost always higher than those for free ammonia and of the same order as those for glutamine. Rye-grass plants, adequately fertilized with ammonium sulfate, have given values up to 15 mg. per cent of urea N under certain environmental conditions. On the other hand, a few sample the same species over the past 2 years have been found to contain measurable quantity of urea, and it is noteworthy that a proportion these samples showed little or no increase in ammonia when dried.

In order to explore the possibility that urea was responsible in whol in part for this increase, the behavior of tissues artificially enriched w urea was studied. Infiltration as a method of enrichment was used earlier experiments but was later replaced either by culture of detac leaves in dilute urea solution (0.1 to 1.0 per cent) or by heavy fertil tion of intact plants in the field (5 to 10 gm. per plant watered into soil). Uptake of urea was of course rapid in detached leaf cultures, the speed with which urea absorption by the roots was manifested in leaves was somewhat surprising. For example, a rye-grass plant bef fertilization contained 1 mg. per cent of urea N in the leaves; withi

TABLE I

Effect of Culture of Rye-Grass Leaves in 0.5 Per Cent Urea Solutions on NH_3-N Glutamine Amide N, Expressed As Mg. per 100 Gm. Fresh Weight

Period of culture	Ammonia N		Glutamine amide N
	Fresh tissue	Dried tissue	Fresh tissue
hrs			
0	0.7	1.4	3.6
1	1.40	8.5	10.4
2	0.47	14.4	25.8
4	1.05	14.6	37.2
6	2.68	12.5	44.7
24	0.47	23.3	46.5

hour the value had increased to 4 mg. per cent and the concentrat reached a peak of 35 mg. per cent of the fresh weight between 16 and hours. In culture experiments, values for urea N of 45 mg. per c have been recorded after 16 hours.

Behavior of Urea-Enriched Tissues on Drying—When artificially enric tissues are rapidly dried at 80°, the increase in ammonia on drying is v much greater than in any case cited in the previous paper. In Tab are given typical results of ammonia values for fresh and dried rye-g leaves after culture in dilute urea solutions, and parallel glutamine val are included to illustrate the rapid and consistent increase in this metabo under these conditions. The figures demonstrate the rapidity with wh urea is absorbed, the consistently low values for free ammonia in the f leaves, and the large divergence between the ammonia values of fresh dried tissue after the 1st hour of culture.

These results, together with the demonstration that urea is a normal leaf constituent, offer strong presumptive evidence for the hypothesis that urea is the source of the extra ammonia found on drying. The breakdown of urea cannot, however, be explained on the grounds of heat lability, since urea is considerably more stable than glutamine when heated in aqueous solution. An alternative explanation is that the breakdown is enzymatic, and the following evidence is adduced to show that this explanation is reasonable.

The establishment of urease as a normal constituent of leaf tissue was the first requirement, and we have confirmed the findings of earlier workers that urease is widely distributed throughout the plant kingdom. By incubation of fresh leaf macerates with urea in the presence of H_2S for periods up to 1 hour at 37°, every tissue examined has shown considerable urease activity.

It then remained to show that the conditions of drying used in this Laboratory, *viz.* dehydration to 5 per cent moisture level at 80° ± 2° in 35 to 45 minutes, are such that the hydrolysis of urea is possible. From careful study of the tissue during the drying period, it is believed that the course of events is as follows: the stream of air at 80° heats the tissue quite rapidly to a temperature (probably about 60°) at which the cell structure begins to break down; dehydration of the cytolyzing leaves is rapid and the latent heat of vaporization prevents any marked rise of local temperature until drying is almost complete. The conditions during this period of cytolysis at intermediate temperatures are conducive to rapid hydrolysis of urea without too serious a loss of urease through heat inactivation.

Two lines of evidence support this general postulate. The first comes from drying experiments at 50°, at which point there is no obvious cytolysis, and at 80° on enriched leaves which were infiltrated with mercuric chloride to prevent enzyme action. In a typical example, the ammonia values for fresh leaves, leaves dried at 80° (for 35 minutes), leaves dried at 80° in the presence of mercuric chloride, and leaves dried at 50° were 1.23, 6.54, 2.37, and 3.21 mg. per cent respectively. When leaves were cytolyzed with ether before drying, the results at 50° and 80° were practically identical.

The second line of evidence is that leaf tissue dried at 80° still exhibits considerable urease activity, as is demonstrated in the following experiment. Dried and ground leaf tissue containing only 1.0 mg. per cent of urea N was incubated with dilute urea solution for 30 minutes at 37° with a consequent increase in free ammonia N of 4 mg. per cent. The demonstration that tissue urease is not completely destroyed during drying has a further implication with respect to the standard extraction procedure. Normally the ground tissue is extracted with water at 80° 10 minutes (2). We felt that, if the urease survived drying at 80°, ther hydrolysis during extraction with hot water might occur. A co parison was therefore made between extraction of dried tissue in the W ing blendor in the cold, and by the standard hot water procedure, relevant results being shown in Table II. Also included are the fr ammonia values and the results of further incubation for 1 hour at of the extracts prepared at 80° to show that inactivation is still not co plete. The freeze-dried tissue in which presumably no inactivation enzyme took place during drying shows large increases in ammonia, b on extraction and subsequent incubation of the hot water extract.

It is reasonable to assume from these data that urea can be enzym cally hydrolyzed during drying, and, on this hypothesis, it is apparent t the level of ammonia in a dried tissue which contains urea and whic extracted at 80° is the result of at least three factors, *viz.* the concentra of ammonia in the fresh leaf, and the hydrolysis of part of the urea in oven and of a further part during extraction with hot water.

TABLE II

Production of Ammonia from Extracts of Urea-Enriched Tissues, Expressed As per 100 Gm. Fresh Weight

Treatment of tissue	Fresh tissue	Dried tissue		
		Cold water extract	Hot water extract	Hot w extra incub
1% urea culture for 16 hrs	0.70	7.5	18.4	22.
Freeze-dried, 1% urea culture, 16 hrs		0.12	7.35	21.
0.5% urea culture, 16 hrs	1.28	3.1	9.58	14.
Fertilized plant, 16 hrs	0.70	1.90	4.2	6.

The finding that tissue urease is not inactivated by heating fo minutes at 80° in dilute aqueous solution is not in accord with the gener accepted properties of enzymes. In this connection we have found t although an aqueous solution of jack bean urease lost its activity c pletely under these conditions, some potency still remained when hea was carried out in the presence of urea. It may also be assumed that tissue colloids exerted some degree of protection during drying and water extraction.

Effect of Cytolysis and Incubation on Urea-Enriched Tissue—Fur insight into the urea-urease system in leaves is gained from the beha of macerated and cytolyzed leaves on incubation. When tissues macerated in the Waring blendor without any additions other tha and water, the resultant macerate shows little or no urease activity, presumably because of inactivation by oxidation with atmospheric oxygen. This phenomenon is also shown by jack bean urease. A partial reactivation occurs on standing at room temperatures, and production of ammonia by urea-containing tissue occurs slowly after the 2nd hour. If, however, a reducing agent such as H_2S is added during blending, inactivation is prevented and the free ammonia value begins to rise immediately. Cytolyzed leaves behave in a fashion similar to that of macerates in the presence of a reducing agent and the results of incubation of cytolyzed and macerated leaves under a variety of conditions are given in Table III. They may be summarized as follows: (1) Leaf urease is seriously but not quantitatively inactivated by blending, inactivation being prevented by addition of reducing compounds. (2) Cytolyzed leaves and reduced macerates give substantially the same ammonia values on standing for 16 hours. (3) Leaf urease, like jack bean urease, is practically inactive at pH 4.5. (4) Added urea is quantitatively hydrolyzed by cytolyzed leaves and by reduced macerates, while the addition of urease to a heat-inactivated tissue gives substantially the same ammonia value after 16 hours as is given by the same tissue on cytolysis and incubation.

TABLE III

Ammonia N of Macerated and Cytolyzed Leaves after Standing for 16 Hours at Room Temperature, Expressed As Mg. per 100 Gm. Fresh Weight

Procedure No.	Treatment of tissue	Tissue 1	Tissue 2	Calculated
1	Waring blendor macerate	6.55	11.9	
2	Macerate with acetic acid to pH 4.5	1.22	2.34	
3	Macerate with H_2S	26.9	27.8	
4	Ether-cytolyzed tissue	24.3	26.0	
5	Cytolyzed tissue with acetic acid	3.56	3.21	
6	Tissue boiled 3 min.	2.04	3.1	
7	Equal volumes of (6) and (4)	45.9		48.6
8	Cytolyzed tissue + urea (= 12.0 mg. % N)	35.9		36.3
9	As (8), but with acetic acid	3.9	2.5	
10	Tissue boiled 3 min. and urease added	22.5	24.0	

The results offer a reasonable explanation for data reported in the previous paper, where a comparison was made between extracts prepared by cytolysis and pressing and those prepared by blending. The results were consistently higher for the former technique, and it is obvious that at least a partial explanation is the hydrolysis of tissue urea which occurs during the time required for preparation of the extract by the cytolysis method. The results also support the conclusion that of the methods

available for estimation of free ammonia in leaf tissue the most accur
is rapid maceration of fresh tissue in the cold at pH 4.5.

Presence in Leaves of Ammonia Precursors Other Than Urea—The resu
presented thus far are designed to show the presence in leaves of u
and urease, to describe the behavior of leaf tissue containing large c
centrations of urea under various conditions, and, on the basis of t
similarity in behavior between urea-enriched and normal leaves, to of
the explanation that the observed increase in ammonia on drying is d
in part to the enzymatic hydrolysis of urea in the oven. Ammonia
creases, however, cannot be wholly explained in this way. When ur
determinations are made concurrently with ammonia determinations
fresh and dried tissue, it is immediately apparent that other potent
ammonia precursors are present. From the hundreds of samples wh
have been analyzed in an attempt to characterize these materials, we ha
collected in Table IV a series which best demonstrates the effect.

The characteristic feature of the data is that the sum of ammonia a
urea nitrogen for dried is higher than that for fresh tissue. Large increa
of ammonia invariably occur (*cf.* Tables I to III), while there is a co
current increase in urea in all the samples listed. If urea were the s
source of ammonia increase on drying, the rise in ammonia would be b
anced by a corresponding decrease in urea. Table IV also provides furth
evidence for the conclusion in the previous paper that glutamine brea
down cannot account for the discrepancy. It is necessary therefore
postulate a source of ammonia other than urea, and initially it was dif
cult to decide whether the unknown material was breaking down to am
monia directly or by way of urea. From Table IV and from a large co
lection of other data, it appears probable that leaves contain a mater
which yields urea, and we shall refer to this material in the remainder
this paper as the "urea precursor." The effect is particularly well demo
strated in Tissues 5 and 6 of Table IV, where the ammonia of the dri
tissue is considerably greater than the ammonia plus urea of the fre
tissue. Our conclusion is that in these cases the breakdown of precursor
the oven is more important than the hydrolysis of the preformed ur
Tissue 7 is included to show the essentially similar behavior of norm
tissue.

Additional information on this point comes from utilization of the c
served inactivation of tissue urease which occurs in the Waring blend
Extracts of fresh tissue were prepared by blending in the presence and a
sence of H_2S, and analyses were made for ammonia and urea after incub
tion for 5 hours at 37°. The results are shown in Table V and again
will be noted that the normal tissue behaves in essentially the same w
as artificially enriched tissue.

The trend is the same in every case. When H_2S is added before maceration, the ammonia increases markedly on incubation. When H_2S is omitted, the increase is significant, due probably to partial reactivation of the oxidized enzyme on standing, but is very much smaller than when the enzyme is protected from inactivation. The figures in Column 4 are consistently higher than those in Column 6, and we believe that the most probable explanation is that the urea precursor is hydrolyzed enzymatically to urea, and that where urease is inactivated by aeration (Column 4) marked urea formation takes place. This explanation appears to cover the observed facts on drying and incubation, and future work is being planned on the basis that the unknown material breaks down through urea to ammonia.

TABLE IV

Comparison of Ammonia, Urea, and Glutamine Amide N on Fresh and Dried Rye-Grass Extracts, Expressed As Mg. per 100 Gm. Fresh Tissue

Tissue No.	Fresh tissue extract			Dried tissue extract		
	NH_3-N	Urea N	Glutamine amide N	NH_3N	Urea N	Glutamine amide N
1. 1% urea culture, 16 hrs.	0.70	18.7	20.8	11.8	38.2	19.0
2. 1% " " 16 "	0.23	31.2		10.9	35.4	
3. 0.5% urea culture, 16 hrs.	2.45	12.6	17.1	8.7	12.8	12.0
4. 1% urea culture, 16 hrs.	1.40	31.9	26.2	4.9*	43.7*	27.4*
5. Fertilized plant, 16 hrs.	3.98	2.44	12.1	11.7	20.2	15.7
6. Same, 64 hrs.	0.47	0.93	22.9	3.8	3.2	17.1
7. Normal leaves.	1.11	1.63		3.16	1.39	

* This tissue was freeze-dried and extracted at 80° for 10 minutes.

TABLE V

Effect of Incubation of Fresh Rye-Grass Extracts on Ammonia N and Urea N, Expressed As Mg. per 100 Gm. Fresh Tissue

Tissue	Fresh extract by boiling 3 min.		Waring blendor macerate, incubated for 5 hrs.		Waring blendor macerate + H_2S, incubated for 5 hrs.	
	NH_3-N (1)	Urea N (2)	NH_3-N (3)	Urea N (4)	NH_3-N (5)	Urea N (6)
Urea fertilization, 18 hrs.	1.23	5.24	2.45	9.75	7.06	5.64
Same.	0.76	4.94	2.10	10.03	5.78	6.46
No treatment.	0.79	1.78	2.16	2.69	2.86	1.69
0.1% culture, 16 hrs.	1.11	7.41	2.68	12.50	6.77	6.83

On current theories of urea metabolism, the most probable urea precu sor in any organism is arginine, and several attempts have been made, l enzymatic and colorimetric methods, to demonstrate decreases of argini with increases either of urea or ammonia. No correlation has been o tained; arginine values remained relatively constant during the procedur outlined above.

The figures in Column 6 of Table V are of interest from another poi of view. From the ease and rapidity with which added urea is brok down by cytolyzed leaves and by reduced macerates, it would be expect that no urea would be present after 5 hours incubation; *i.e.*, that the valu in Column 6 would be zero. This is never the case, while an exact parallel observation has been made on many plant extracts which ha been incubated with jack bean urease. For example, a tissue containi originally 0.24 mg. of ammonia N and 5.76 mg. of urea N per cent w macerated in the presence of H_2S and incubated with urease for 2 hou when the corresponding figures were 1.81 and 4.44 mg. per cent respe tively. Thus, despite the presence of relatively excessive amounts urease during the period of incubation, the addition of fresh urease f the residual urea determination has brought about further productio of ammonia. Moreover, when urea was added to the macerate in quant ties greater than that present in the tissue, it was quantitatively recovere as ammonia after 2 hours. This last finding might be explicable on th ground that jack bean meal contains amidases other than urease, were not for the observation that crystalline urease behaves in the same ma ner. No explanation can be offered for this anomalous behavior, whic is reported to emphasize the complexity of the systems leading to th production of ammonia in green leaves.

Effect of Environmental Conditions—It has already been mentioned tha the phenomena under investigation, which hinge around urea and relate compounds, manifest themselves in greatly varying degree throughout th year. There are seasons of the year when no urea or urea precursor ca be found in rye-grass and when there is no significant increase in ammoni when the tissue is dried. At certain times of the year, heavy fertiliz tion of rye-grass plants with urea causes little or no rise in urea conce tration and no apparent production of urea precursor, even though the is a rapid increase in other soluble nitrogenous components, indicati that the urea is being absorbed. Moreover, if leaves from similar plan are cultured in dilute urea solutions, the leaf urea level rises as usual b there is no production of urea precursor. The change-over from one ty of utilization mechanism to the other is illustrated in Table VI, where tw apparently identical rye-grass plants derived from the same parent cl were heavily fertilized with urea on October 27 and November 1 respe tively, and analyzed on October 28 and November 2. It can be seen th within the short space of 5 days a very different picture is obtained. This is not an isolated finding, since no tissue analyzed between July and about the end of October showed any significant production of urea precursor under urea culture or fertilization, whereas from November until April the majority of rye-grass plants behave similarly to the second one shown in Table VI. This latter generalization holds true, except under dry hot conditions which may cause cessation of growth during January and February, and before the autumn rains, which in New Zealand normally fall during March.

The rate of growth is certainly not the governing factor in the change from one type of metabolism to the other, since rye-grass under our conditions grows at least as rapidly during August and September as in November. From our observations for over nearly 3 years, we have come to the conclusion that urea metabolism follows substantially different pathways at different seasons of the year, which is equivalent to concluding that the enzymatic complex is subject to considerable variation.

TABLE VI

Differential Response of Rye-Grass Plants to Urea Fertilization

The results are expressed as mg. per 100 gm. of fresh tissue.

1948	Fresh tissue		Dried tissue	
	Ammonia N	Urea N	Ammonia N	Urea N
Oct. 28	0.62	1.36	3.15	1.23
Nov. 2	0.64	19.6	6.25	31.5

DISCUSSION

The high levels of urease in certain seeds, established between 30 and 40 years ago, together with subsequent investigations showing the wide distribution of urease throughout the plant kingdom, have naturally led to speculation about the part played by the urea-urease system in plant metabolism. The presence of urea in the vegetative portions of higher plants was first satisfactorily shown by Fosse (3), but, through his use of the non-specific xanthydrol reagent, he was unable to distinguish between free and combined urea. Klein and Taubock (4), using the urease method, were able to show beyond doubt that free urea occurs in higher plants, although they were able to demonstrate its presence only in actively metabolizing tissues. From numerous experiments on seedlings grown in solutions of arginine, Klein and Taubock concluded that the greater part of the urea of higher plants results from the hydrolysis of arginine.

Since these publications, the last of which appeared in 1933, we have found only one paper dealing with urea concentrations and ur metabolism in plants, *viz.* that of Damodaran and Venkatesan (5), whic appeared while this paper was in course of preparation. These investig tors showed that, during the germination of *Dolichos biflorus* and *Phase lus mungo*, urea concentrations rose sharply and reached high levels b tween the 10th and 20th days. Simultaneous arginine determinatio led to the conclusion that only part of the urea could be accounted for b decrease in arginine. Apart from this paper, no attention appears to hav been paid to urea during the past 15 years and the reviews of the nitrog enous metabolism in plants published during that period make onl passing reference to urea, the whole emphasis in ammonia metabolis being placed on asparagine and glutamine.

It is not suggested that the data presented in this paper in any way in validate the conclusions reached by Vickery, Chibnall, and their co workers as to the key position of asparagine and glutamine in plan nitrogen metabolism; but the demonstration both in this paper and b Damodaran and Venkatesan of the presence in plants of urea justifi its inclusion as a third amide in the nitrogen cycle.

No specific information can be offered as to the part played by urea i nitrogen metabolism. The rapid uptake of urea by intact plants throug the roots and the utilization of this urea to form glutamine ma mean nothing more than that, if urea is absorbed by the plant, the urea urease system can act as an internal source of ammonia. On the othe hand absorption through the roots does not explain the presence of ure to the extent of 1 to 5 mg. per cent in a wide variety of plants which hav received no urea fertilization; nor does it explain the fact that rye-gras well fertilized with ammonium sulfate may contain up to 15 mg. per cen of urea under suitable environmental conditions. The observed phenom ena may be explicable on the assumption of the operation of a Kreb ornithine cycle, in which case urea would arise from hydrolysis of arginine Free arginine exists in the plant sap, and, even though the concentratio is low,[1] there is a large reserve in the leaf protein which could be drawn o as required.

That this is not the complete explanation is seen from Tables IV an V, as well as from the studies of Fosse and of Klein and Taubock. Th evidence is based largely on the behavior of artificially enriched leave but it must be stressed that the purpose of enrichment was solely to exag gerate and define clearly an effect which is also given by normal leave There is no reason to suppose that normal leaves do not follow the sam metabolic course at a lower level.

Serious consideration does not appear to have been given to the poss bility that urea may act as a raw material for synthesis in biological systems without the necessity of first being hydrolyzed to ammonia. Two lines of evidence point to this possibility in plants. Firstly, the urea precursor reaches higher levels under urea culture and fertilization than are reached when ammonia is used. Secondly, there are consistent and, we believe, significant differences in glutamine values for plants which receive their nitrogen as urea and ammonia respectively. If urea were acting only as a source of ammonia, it would be expected that no differences would be apparent in either case.

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[1] Bathust, N. O., private communication.

The postulate that the increase in ammonia on drying comes from the hydrolysis in the oven of urea and of a urea precursor is at variance with the conclusion reached in the previous paper (1); *viz.*, that there exists in leaves an ammonia precursor which is more labile than glutamine. Since our recognition of the possibility of enzymatic activity during drying, no evidence for a heat-labile ammonia precursor has been obtained and the earlier conclusion is therefore withdrawn.

Apart from the importance of the establishment of urea as a plant metabolite, there is a further point of interest in studies of heavy urea fertilization. Animal production in New Zealand is almost entirely based on pastures, and investigations of the effect of the grazing animal on the growth of pasture have been in progress for a number of years (6). It has been shown that grazing animals return up to 500 pounds of nitrogen per acre in dung and urine, and that of this amount over 50 per cent is in the form of urea. The concentration of urea in a single urine patch is high and an investigation is in progress as to the proportion of this urea which is absorbed as such by the pasture plants, the proportion which is absorbed as ammonia arising from hydrolysis of the urea, and the proportion which is oxidized to nitrate and absorbed in that form.

The technical assistance of Miss J. L. Fisher and Miss R. N. Haycock is gratefully acknowledged. Thanks are also due to Dr. J. B. Sumner for gifts of crystalline urease and of jack beans, and to the Director and staff of the Grasslands Division, who have supplied the raw material on which this investigation was based.

SUMMARY

Urea and urease are present in significant concentrations in a series of plants, and the former hydrolyzes enzymatically during drying at 80° to give ammonia. Evidence is also presented for the presence under certain environmental conditions of a urea precursor which hydrolyzes to give first urea and finally ammonia when leaves are dried in the oven or incubated after cytolysis with ether.

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THE EFFECT OF D-TRYPTOPHAN ON THE UTILIZATION OF THE L ISOMER BY SOME LACTIC ACID BACTERIA

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The utilization of the isomers of tryptophan by *Lactobacillus arabinosus* 7-5 has been studied by several investigators. Greene and Black (1, 2) oncluded that D-tryptophan was not utilized by this organism. The procedure outlined for the determination of tryptophan by these investigators was based on the interpretation that DL-tryptophan is exactly one-half as active as the pure L isomer. Wooley and Sebrell (3) found two amples of DL-tryptophan which had exactly 50 per cent of the activity of heir L-tryptophan, and one sample which exhibited 65 per cent activity. Baumgarten *et al.* (4) reported that twice the amount of DL-tryptophan was required to produce the same amount of acid as that produced by the L form. Greenhut *et al.* (5) found two samples of DL-tryptophan which possessed one-half the activity of samples of L-tryptophan. A sample of D-tryptophan showed no activity.

The inactivity of D-tryptophan for *Streptococcus faecalis* R is indicated n the reports by Stokes *et al.* (6), Baumgarten *et al.* (4), and Kuiken *et al.* (7).

During tests on tryptophan samples carried out by the present authors or the purpose of selecting standards, it was found that when tested at igh levels several samples of DL-tryptophan had approximately one-half he activity of the L isomer, but at low levels, the activity of the DL compound was considerably less. These experiments pointed to an inhibitory effect of the D compound on the utilization of the L form. More definite information was desired concerning this inhibition than could be obtained in experiments in which the activities of L and DL compounds were compared. Pure D-tryptophan was therefore prepared, and its effect on the utilization of L-tryptophan by several lactic acid bacteria was tudied. The purpose of this paper is to report the results of these tests.

An inhibitory effect of high concentrations of D-leucine and D-valine on he growth of *Lactobacillus arabinosus* (8, 9) and the inhibition of *Escherichia coli* (10) by D-alanine, D-leucine, and D-valine has been studied

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by Fox, Fling, and associates. The concentrations of the unnatural an
acids used in these investigations were much higher than those norm
encountered in media for the microbiological determination of an
acids.

EXPERIMENTAL

D-Tryptophan was prepared by the resolution of DL-tryptophan acc
ing to the method of Berg (11). The value obtained for the spe
rotation of the acetyl-D-tryptophan quinine salt obtained by fracti
crystallization was $[\alpha]_D^{25} = -121.0°$. Berg reported $[\alpha]_D^{20} = -12$
The D-tryptophan prepared had a nitrogen content of 13.48 per
(calculated 13.72).

In order to make certain that the results obtained were not influen
by the type of medium used, the tests were carried out with media cont
ing (*a*) purified amino acids (Kuiken *et al.* (12)), (*b*) acid-hydroly
casein (Riesen *et al.* (13)), and (*c*) hydrogen peroxide-treated pept
(Kuiken *et al.* (7)). The general procedures used for conducting the t
were the same as given in the above reports. The same type of res
was obtained by the use of all three kinds of media.

Table I gives the results of tests on the activity of a number of tryp
phan samples with three different organisms. The activity of the sam
was referred to L-tryptophan, Lot 1, as a reference. In these tests me
containing acid-hydrolyzed casein were used. It will be noted that
relative activity of the DL compounds as determined by *Streptococ*
faecalis R was not the same as the activity determined by *Lactobaci*
arabinosus 17-5 or *Leuconostoc mesenteroides* P-60.

The D-tryptophan prepared by resolution of the DL compound sho
no activity when tested alone with *Lactobacillus arabinosus*, *Streptoco*
faecalis R, and *Leuconostoc mesenteroides*.

With *Streptococcus faecalis*, the addition of equal amounts of D-tryp
phan to L-tryptophan had no effect on the utilization of the natural c
pound. With *Lactobacillus arabinosus* and *Leuconostoc mesenteroides*
results were quite different. The inhibitory effect of the D-tryptop
on the growth of *Lactobacillus arabinosus* is shown in Fig. 1. The
culated recovery of the L-tryptophan in the presence of an equal amo
of the D isomer was 60 per cent at a level of 1 γ per tube, 81.2 per
at a level of 4 γ per tube, increasing to 85.5 per cent at a level of 9 γ
tube. When L-tryptophan was tested in the presence of five times
amount of D-tryptophan, the inhibition was somewhat greater. The
culated recovery of the L-tryptophan at a 1 γ level was 43 per cent,
at a 4 γ level it was 73.5 per cent. It will be noted that the growt
sponse with equal quantities of D- and L-tryptophan was very nearly
same as with DL-tryptophan.

TABLE I

*Per Cent Activity of Various Tryptophan Standards**

Tryptophan isomer	*S. faecalis*		*L. arabinosus* (Texas)		*L. arabinosus* (Wisconsin)†		*L. mesenteroides*	
	No. of tests	Average activity	No. of tests	Average activity	No. of tests	Average activity	No. of tests	Average activity
L, Lot 1	5	100.0	4	100.0	2	100.0	3	100.0
" " 2	4	97.6	3	97.1	2	99.8	3	98.4
" " 3	4	100.3	2	99.6	2	100.0	3	97.5
DL, " 1	4	46.0	2	39.1	2	34.3	3	39.6
" " 2	4	50.0	2	39.9	2	37.8	3	43.0
" " 3	7	49.5	4	42.1	4	42.5	6	44.6
" " 4	5	48.5	4	41.6	2	38.4	3	38.7

* No. of tests refers to separate assays with eight or more tubes included in each assay at four or more test levels. The levels tested are summarized for the tests in which 2.5 to 10 γ of L-tryptophan were used or double this amount of DL-tryptophan.

† Stock culture of *Lactobacillus arabinosis* obtained **through the courtesy** of Dr. H. E. Sauberlich, University of Wisconsin.

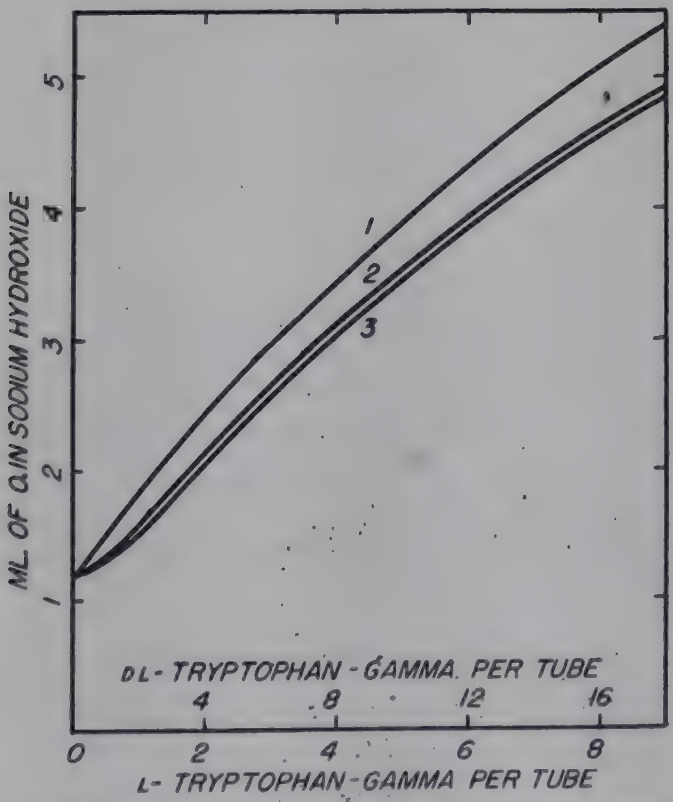

FIG. 1. The effect of D-tryptophan on the utilization of the L isomer by *Lactobacillus arabinosus* 17-5. Titration values are for 5 ml. aliquots from 10 ml. cultures. *1*, L-tryptophan; *2*, L-tryptophan plus an equivalent amount of D-tryptophan; *3*, DL-tryptophan.

In tests with *Lactobacillus arabinosus* with a medium containing 1 mg. of L-tryptophan per tube, no inhibition of growth at all was produced by

4, 16, 50, or even 100 γ of D-tryptophan. This finding substantiates t interpretation that the inhibitory effect is due to interference with t utilization of the natural compound.

Fig. 2 shows the results of similar tests with *Leuconostoc mesenteroid* In this case, the mixture of D- and L-tryptophan showed exactly the sa activity as the sample of DL-tryptophan. The extent of inhibition p duced by the presence of the D compound varied considerably with t different test levels. The calculated recovery at a level of 4 γ of L-tryp phan was only 60 per cent. At a level of 16 γ, no inhibition was evidenc

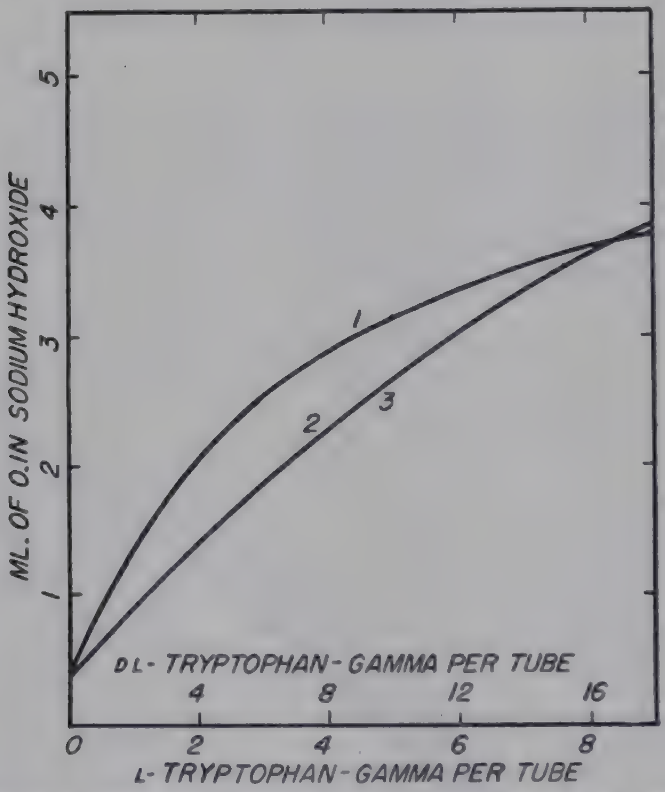

FIG. 2. The effect of D-tryptophan on the utilization of the L isomer by *Leucon* *toc mesenteroides* P-60. Titration values are for 5 ml. aliquots from 10 ml. cu tures. *1*, L-tryptophan; *2*, L-tryptophan plus an equivalent amount of D-trypt phan; *3*, DL-tryptophan.

In some, but not in all of the experiments, a slight utilization of D-trypt phan by *Leuconostoc mesenteroides* at the higher test levels was indicate

DISCUSSION

In view of the findings presented in this paper, it is apparent that t proper selection of a standard to be used in microbiological assays f tryptophan will depend on the organism used and on whether the trypt phan in the unknown is in the L or DL form. For assays with *Lactobacill arabinosus* or *Leuconostoc mesenteroides*, L-tryptophan should be used as standard when the samples are hydrolyzed enzymatically, and the DL form should be used when alkaline hydrolysis is employed. With *Streptococcus faecalis* R, L- and DL-tryptophan can be used interchangeably as standards on the basis that the DL compound is exactly one-half as active as L-tryptophan.

It is probable that the inhibitory effect of the unnatural isomer on *Lactobacillus arabinosus* has been previously overlooked because the inhibition becomes much less apparent when the higher levels of DL-tryptophan are used. Differences in the composition of the media or in the behavior of the organism are probably not involved, since in the present investigation three different media and two different *Lactobacillus arabinosus* cultures were employed.

Microbiological methods have proved very effective for the detection of small amounts of impurities in amino acid samples. It has been the experience of the authors that particular care should be exercised in the selection of tryptophan standards, aside from the choice of the isomer to be used. By the use of microbiological techniques, several samples of tryptophan which had been assumed to be pure were found to contain only 95 to 97 per cent tryptophan.

The finding that the equivalence of DL-tryptophan in terms of L-tryptophan for *Lactobacillus arabinosus* is dependent on the test level is in agreement with the results of Holland and Meinke.[1]

SUMMARY

Contrary to previous reports, it was found that DL-tryptophan had less than one-half the activity of the L compound for *Lactobacillus arabinosus*. The equivalence of the DL compound in terms of L-tryptophan varied with the test level, approaching 50 per cent at the higher levels.

Pure D-tryptophan was prepared by resolution of the DL compound and its effect on the utilization of L-tryptophan by *Lactobacillus arabinosus* 17-5, *Leuconostoc mesenteroides* P-60, and *Streptococcus faecalis* R was studied. The unnatural isomer showed an inhibitory effect on the growth of the first two of these organisms when tested at low concentrations of the L compound.

D-Tryptophan had no effect on the growth of *Streptococcus faecalis* R under any of the conditions investigated.

This investigation was supported in part by a grant from Swift and Company.

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[1] Holland, B. R., and Meinke, W. W., unpublished data.

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THE EFFECT OF POTASSIUM IONS ON THE ABSORPTION OF ORTHOPHOSPHATE AND THE FORMATION OF METAPHOSPHATE BY BAKERS' YEAST*

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(Received for publication, August 4, 1948)

Jeener and Brachet (1) found that the absorption of inorganic phosphate by bakers' yeast is greatly enhanced when the incubation of the yeast in a phosphate-containing nutrient (period of P absorption) is preceded by a period of incubation in a medium devoid of phosphate (period of P starvation). The Belgian authors had already demonstrated that a large part of the absorbed phosphate appears in the yeast cells as a compound which is different from orthophosphate. Wiame (2, 3) and independently Schmidt, Hecht, and Thannhauser (4) characterized this compound as a metaphosphate.

Observations concerning the occurrence of metaphosphate in yeast and molds have previously been reported in the literature. Kossel (5) and Ascoli (6) found in the nucleic acid fraction of yeast a compound resembling metaphosphate. More than 40 years later MacFarlane (7) conclusively identified this substance as a metaphosphate. Mann (8) isolated a metaphosphate from *Aspergillus*.

The rates of phosphate assimilation by bakers' yeast are little affected by varying the pH values of the medium between 4 and 7.5 or by varying the phosphate concentrations between 0.002 and 0.1 M. Variations of the mutual proportions of the cations as well as the complete omission of sodium and magnesium salts during the period of phosphate absorption likewise had little influence on these processes.

It was found, however, that the absorption of orthophosphate and the formation of metaphosphate by the yeast were strongly inhibited in media which were devoid of potassium salts. In the present paper, we will describe experiments in which this effect of potassium deficiency was studied.

EXPERIMENTAL

Material—All experiments were carried out on samples of Fleischmann's bakers' yeast, each of which was obtained from Standard Brands Incor-

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* This study was aided by grants from the Rockefeller Foundation, the American Cancer Society, the Godfrey H. Hyams Trust Fund, the Bingham Associates Fund, and the Charlton Fund.

porated, Cambridge, Massachusetts, on the day of its arrival. The b havior of these yeast samples was usually well reproducible in regard to th absorption of orthophosphate, the formation of metaphosphate, and the rat of fermentation and respiration. (Yeast samples obtained from grocer stores differed widely in these respects and were never used in the exper ments described in this paper.)

Nutrient Media—In all experiments the yeast samples were aerated i 100 volumes of the nutrient solutions, prepared by modifying a "bas medium" which contained (per liter) 55.5 mM of glucose, 100 mM of sodiu succinate buffer (pH 5.0), 38.2 mM of ammonium sulfate, 9.4 mM of pota sium chloride, and 2.9 mM of magnesium sulfate.

Methods—Sodium, potassium, and magnesium were determined a sodium uranyl zinc acetate, potassium chloroplatinate, and magnesiu ammonium phosphate respectively (9, 10). The ash obtained by heatin the samples in platinum crucibles at 400° in an electric oven for 5 hou was hydrolyzed in 3 N hydrochloric acid in a boiling water bath for hour, evaporated to dryness on the water bath, redissolved in 2 cc. of hydrochloric acid, and brought to a volume of 10 cc. Aliquots of the solutions were used for the determinations of the metals. The P dete minations were carried out according to the method of Fiske and Subbaro (11).

Influence of Cations (Potassium, Magnesium, Sodium, and Ammoniu on Absorption of Orthophosphate by Bakers' Yeast

The results of representative experiments are shown in the curves Fig. 1.[1] Curves I and II were obtained on aliquots of the same yea suspension which were incubated simultaneously in the different medi It can be seen that the omission of potassium ions from the mediu (Curves I and II) largely prevents the absorption of phosphate. Th differences between the final amounts of the absorbed phosphate in th presence (Curves A) and absence (Curves B) of potassium would ha been approximately 3 times larger if media of higher phosphate conte had been used for the experiments.[2]

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[1] All curves in Figs. 1 to 4 represent exclusively the behavior of the yeast samp during the period of the phosphate absorption. The data concerning the cond ions of the preliminary periods of phosphate starvation are included in each leger but the behavior of the yeast samples during the phosphate starvation periods not shown in the curves.

[2] Media with low concentrations of phosphate were chosen for the experime with the purpose of avoiding the necessity of determining relatively small diff ences between large absolute amounts of phosphate. The time curve of the ph phate absorption of phosphate-starved bakers' yeast levels off after 2 hours wh potassium ions are present in the medium. The term "final amounts of absorb phosphate" refers to the amounts absorbed during the duration of the rapid ph of the process.

In the experiments of Curves I_A and I_B, potassium was absent during ne period of the phosphate starvation, as well as during that of absorpion, while in experiments of Curves II_A and II_B the potassium-containng basic medium was used during the period of phosphate starvation.

The effects of potassium deficiency are similar in both experiments. he somewhat stronger influence in the first case is presumably due to the act that the yeast cells lose appreciable quantities of potassium during he periods of phosphate starvation in media devoid of potassium.

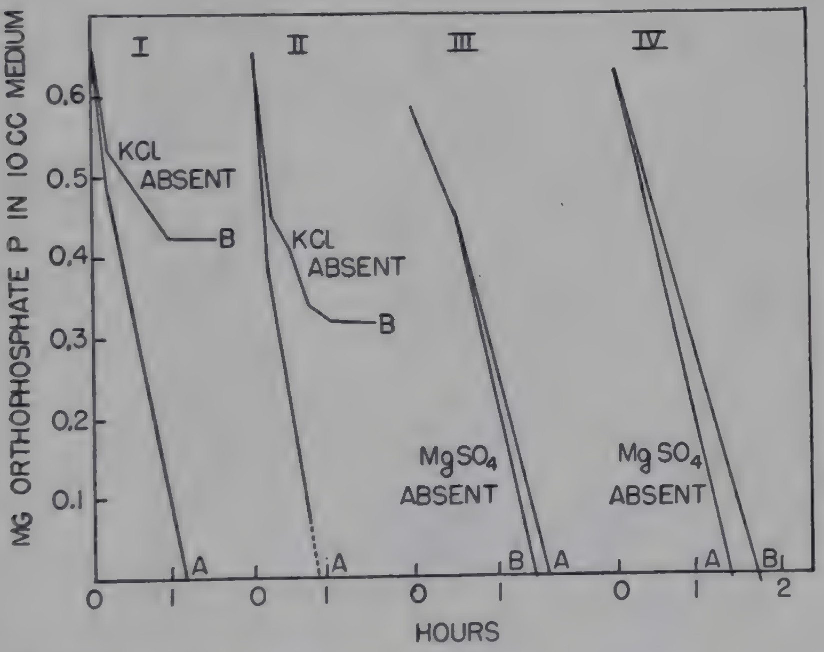

FIG. 1. The absorption of phosphate by bakers' yeast from media devoid of po- assium (Curves I and II) and of magnesium (Curves III and IV). Period of phos- hate starvation, all media were devoid of phosphate; samples for Curves I and III, ncubated in the basic medium; sample for Curve II, in the basic medium devoid of K^+; and for Curve IV, in the basic medium devoid of Mg^{++}. Period of phosphate absorption, all media contained (per liter) 2 mM of NaH_2PO_4; the yeast samples of Curves I_A, II_A, III_A, and IV_A were incubated in the phosphate-supplemented basic medium, the samples of Curves I_B and II_B in the phosphate-supplemented basic medium devoid of K^+, and the samples of Curves III_B and IV_B in the phosphate-supplemented basic medium devoid of Mg^{++}.

Fig. 2 shows the influence of different potassium concentrations on the phosphate absorption. The maximal effect was obtained with a potassium concentration of 10 mM, but even a concentration of 5 mM had a strong enhancing influence.

The absence of magnesium (Fig. 1, Curves III and IV) or sodium ions from the media had no appreciable influence on the phosphate absorption.

Ammonium ions had an enhancing effect on the phosphate absorption of bakers' yeast, particularly when the period of phosphate starvation had been omitted (Fig. 3). The curves of Fig. 1 demonstrate that, despite this effect, the inhibition of the phosphate absorption in potassium- ficient media is not abolished by the presence of ammonium ions.

In contrast to the behavior of brewers' yeast (12, 13), the enhanc influence of ammonium ions cannot be attributed to their indispensabil for growth, since the uptake of phosphate during the first 2 hours of cubation is more than 10 times higher than the amount of nitrogen assi ilated.

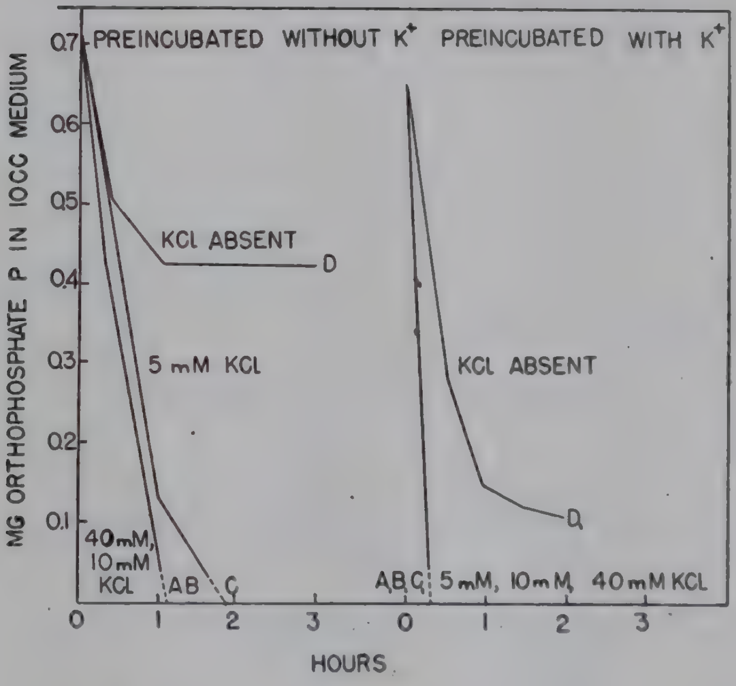

FIG. 2

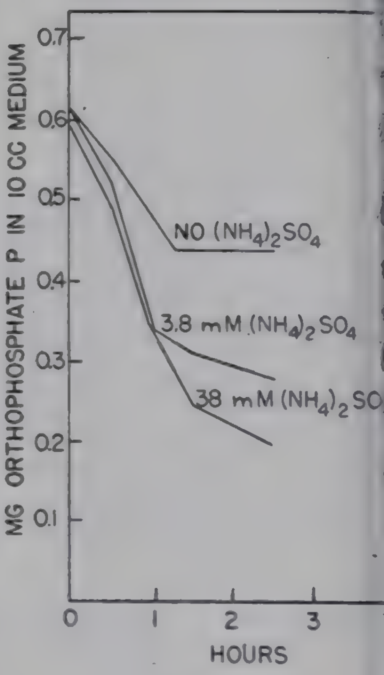

FIG. 3

FIG. 2. Influence of various concentrations of KCl on the absorption of orth phosphate by phosphate-starved bakers' yeast. Period of phosphate starvatic Curves A, B, C, and D, KCl absent in all media; Curves A_1, B_1, C_1, and D_1, KCl pr ent in all media. Period of phosphate absorption, KCl concentration of the mec used in Curves A and A_1, 40 mM; Curves B and B_1, 10 mM; Curves C and C_1, 2 m Curves D and D_1, 0; all media contained 2 mM of NaH_2PO_4 per liter.

FIG. 3. Influence of various concentrations of ammonium ions on the absorpti of orthophosphate by fresh bakers' yeast. All media contained 2 mM of NaH_2P per liter. Except for the concentrations of monosodium phosphate and of a monium sulfate, the composition of the media corresponded to that of the ba medium.

It appeared possible that the effect of potassium on the absorption phosphate was only a secondary consequence of its effect on the ener metabolism. We found, however, that the addition of phosphate to t medium, either in the presence of potassium chloride (10 mM per liter) in its absence, was followed by immediate increases of the rates of ferment tion. The increase was approximately 25 per cent higher in the presen of potassium than it was in its absence. Nevertheless, even in the absen of potassium ions the rate of fermentation was far in excess of that required as a source of energy for the formation of the accumulated amounts of metaphosphate. It is therefore not likely that the stoppage of the absorption of orthophosphate in the absence of potassium is caused by the slower rate of the fermentation.

A comparison between Curves I_A and II_A of Fig. 1 shows that the enhancing effect of potassium ions on the absorption of phosphate is only slightly impaired by the absence of potassium ions during the period of phosphate starvation. On the other hand, the absence of potassium ions during the period of phosphate absorption irreversibly impairs the phosphate-absorbing power of the yeast cells. Fig. 4 shows that the subsequent addition of potassium chloride to a suspension of phosphate-starved yeast which had ceased to absorb phosphate from a potassium-deficient phosphate solution induces only a very sluggish resumption of the phosphate absorption. Similar observations were made on fresh yeast. It appears that the lack of potassium affects the phosphate-absorbing power

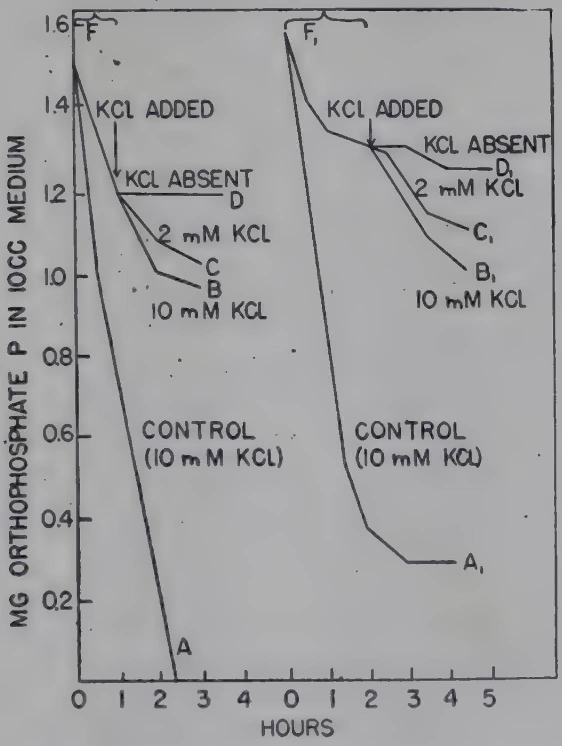

FIG. 4. Early ceasing of phosphate absorption by phosphate-starved bakers' yeast in media devoid of K^+; its resumption after addition of KCl. Curves A, B, C, and D, phosphate starvation in presence of KCl; Curves A_1, B_1, C_1, and D_1, phosphate starvation in absence of KCl. Period of phosphate absorption, Curves F and F_1, media devoid of KCl; after the phosphate absorption had ceased (indicated by the arrows), KCl was added to three equal aliquots of the yeast suspension corresponding to 10, 2, and 0 mM of KCl per liter respectively (Curves B and B_1, C and C_1, D and D_1). Concentration of orthophosphate in medium, 5 mM.

of the yeast to a much higher degree in the presence of phosphate than does in its absence.[3]

TABLE I

Coupling of Absorption of Cations and of Orthophosphate by Phosphate-Starve Bakers' Yeast; Preferential Absorption of K Ions from "Complete" Medium of Low Sodium Concentration (2 mM of NaH_2PO_4)

Starvation period	Condition of yeast	Absorbed orthophosphate, mM* per gm. moist yeast	M.eq. per gm. moist yeast		
			K$^+$	Mg^{++}	Na$^+$
	Fresh		0.126	0.045	
K$^+$-free medium	After P starvation (medium devoid of K$^+$)		0.095	0.039	0.06
	After P absorption (medium devoid of K$^+$)	0.073	0.082	0.061	
	After P absorption (medium containing K$^+$)	0.210	0.268	0.122	0.03
K$^+$-containing medium	After P starvation (medium containing K$^+$)		0.170	0.051	
	After P absorption (medium devoid of K$^+$)	0.100	0.120	0.085	0.028
	After P absorption (medium containing K$^+$)	0.210	0.250	0.127	

* The increments have been expressed in millimoles of phosphate and milliequiv lents of cations for the purpose of the comparison between the accumulation phosphate compounds and that of the cations in the yeast. It is obvious that the data do not permit any conclusions concerning the osmotic conditions in the yea cells, since it has been shown by Wiame (3) and by the authors (4) that 60 to 80 p cent of the accumulated phosphate compounds consists of highly polymerized met phosphate complexes. The degree of polymerization is at least that of a hexamet phosphate.

Absorption of Cations by Bakers' Yeast during Absorption of Phosph

It was shown by Schmidt, Hecht, and Thannhauser (4) that a large pa of the metaphosphate accumulated in the yeast cells can be extract with water after the yeast has been dried with alcohol and ether.[4] The acidity of such extracts, which contain only negligible amounts of proteins, is always close to pH 6 and does not essentially differ from that of the yeast before the accumulation of metaphosphate. This can only be explained by the assumption that the accumulation of metaphosphate in the yeast cells is accompanied by that of an approximately equivalent amount of cations of low molecular weight.

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[3] The additions of potassium chloride were carried out in different ways: by ac ing solutions of potassium chloride alone or mixtures of potassium chloride a glucose to the incubation yeast or by transferring the yeast cells to fresh, potassi chloride-containing media. In all experiments the results were similar to th shown in Fig. 3. This excludes the possibility that the incomplete restoration the absorption of orthophosphate by potassium chloride might have been cau by the exhaustion of the available amounts of glucose or by the accumulation inhibitory metabolites in the medium.

TABLE II

Coupling of Absorption of Cations and of Orthophosphate by Phosphate-Starved Bakers' Yeast; Preferential Absorption of K Ions from "Complete" Medium of High Sodium Concentration (100 mM of NaH_2PO_4 per Liter); Preferential Absorption of Na Ions and Lack of Absorption of K Ions from Mg-Deficient Medium of High Sodium Concentration (100 mM of NaH_2PO_4 per Liter)

Experiment B, period of P starvation; yeast aerated for 16 hours in 100 volumes of a nutrient solution containing 55.5 mM of glucose, 100 mM of sodium succinate buffer, pH 5, 38.2 mM of $(NH_4)_2SO_4$, 9.4 mM of KCl, and 2.9 mM of $MgSO_4$ per liter. Experiment C, period of P absorption; after treatment according to Experiment B, the yeast is transferred to 100 volumes of a nutrient solution containing 55.5 mM of glucose, 100 mM of NaH_2PO_4, 39.2 mM of $(NH_4)_2SO_4$, 9.4 mM of KCl, and 2.9 mM of $MgSO_4$ per liter. Experiment C_1, period of phosphate absorption in absence of Mg ions; treatment of the starved yeast as in Experiment C_1, except that the medium does not contain $MgSO_4$.

Experiment	Condition of yeast	mM per gm. yeast		M.eq. per gm. moist yeast				
		Inorganic P	Total P	K^+	Mg^{++}	Na^+	Non-protein N	NH_3
A	Fresh yeast	0.023	0.039				0.224	0
B	After P starvation	0.019	0.035	0.134	0.028	0	0.352	0.035
C	" " absorption in presence of Mg	0.148	0.442	0.316	0.325	0	0.415	0.046
C_1	After P absorption in absence of Mg	0.155	0.475	0.140	0.039	0.336	0.790	0.086

Tables I and II show the total amounts of potassium, magnesium, and sodium ions of the yeast before and after its incubation in phosphate-containing media. The analyses reported in Table I were carried out on

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[4] In one experiment, corresponding samples of the yeast in various stages of P starvation and P absorption were extracted respectively with water and with 7 per cent trichloroacetic acid. The results obtained on the acid extracts were similar to those obtained with the water extracts. The yields of all fractions (with the exception of the inorganic P) of the acid extracts, however, were approximately 75 per cent of those obtained in the water extracts.

aliquots of the yeast samples of the experiments shown in Fig. 1, Cu
I and II, and are thus directly comparable with the P values recorde
these curves at the end of the experiments. Table II contains the res
obtained in the presence of a high (0.1 N) concentration of primary sod
phosphate. In this experiment the absorption of phosphate by the y
was measured by determinations of the total P of the washed yeast at
beginning and the end of the incubation periods.

The data of Tables I and II demonstrate that the absorption of ph
phate by the yeast is accompanied by the uptake of very considera
amounts of cations, in particular of potassium ions, from the media.
the presence of magnesium (Table I; Table II, Experiment C), the
sorption of potassium ions far exceeds that of sodium ions, regardles
the mutual proportion of K to Na in the nutrient media, which was
in the experiments recorded in Table I, but only 0.11 in that of Tabl
(Experiment C). A similar preference of bakers' yeast for potassium i
was observed in magnesium-deficient media in which the concentratio
K ions exceeded that of Na ions (K:Na = 4.5).

When, however, the absorption of phosphate by bakers' yeast
studied in a Mg-deficient medium of high sodium concentration (Na^+,
mM; K^+, 9.4 mM) (Table II, Experiment C_1), it was found that practicall
potassium but very considerable amounts of sodium ions were absorb
This result was consistently observed in five analogous experiments with
ferent samples of yeast. It shows that, under certain conditions, la
amounts of phosphate are absorbed by bakers' yeast without a simultane
absorption of potassium ions. Hence, the striking differences betw
the amounts of absorbed phosphate in the presence and those in the
sence of potassium ions cannot be explained by the assumption that
yeast must use potassium ions for the neutralization of the accumula
phosphate groups.

DISCUSSION

Absorption of Phosphate and Formation of Metaphosphate—Since de
minations of orthophosphate and of the total phosphate were used
experiments concerned with the phosphate exchange between the nutr
media and the yeast, the strict interpretation of the results appears t
limited to the absorption of phosphate. In earlier investigations (2,
however, it has been demonstrated that the larger part of the orthop
phate absorbed by phosphate-starved bakers' yeast is rapidly transfor
into metaphosphate. This is also true for the orthophosphate abso
by fresh commercial bakers' yeast during the first 2 or 3 hours of incuba

The formation of metaphosphate has so far been demonstrated onl
living yeast with the orthophosphate of the nutrient medium as the r

urce for the formation of metaphosphate. It is not possible, therefore, decide whether the effects of potassium ions described in this paper in- lve the penetration of orthophosphate into the cells or the chemical echanism of the metaphosphate formation. This question can be an- vered only when it becomes possible to study the formation of meta- osphate in cell-free enzyme preparations.

Physiological Significance of Metaphosphate—No evidence exists in favor a possible rôle of metaphosphate as a direct phosphate donor in enzy- atic transphosphorylations. So far, our experiments in this direction mammalian tissues as well as on yeast have given negative results. akers' yeast, however, contains considerable amounts of an enzyme which ydrolyzes metaphosphate to orthophosphate. Since this reaction liber- tes amounts of energy similar to those produced during the hydrolysis of yrophosphates, it appears possible that one of the biological functions of etaphosphate is that of an energy transmitter. Our information con- erning the functions of both metaphosphatase and of adenylpyrophos- hatase is as yet limited to the knowledge of the exothermic nature of oth enzyme reactions. Experimental proof for the utilization of the nergy generated during the hydrolyses of the polyphosphates is still cking.

SUMMARY

1. The absorption of orthophosphate and the formation of metaphos- hate by bakers' yeast is accompanied by the absorption of cations from he medium. Since the amounts of cations absorbed are sufficient to eutralize the amounts of ortho- and metaphosphate which are accumulat- ng in the yeast cells, it is concluded that only a small part, if any, of the etaphosphate formed exists in the yeast cells in a protein-bound form.

2. The different cations of the medium are not indiscriminately absorbed y the yeast cells. A strong preference for the absorption of potassium ons was found under many conditions. In magnesium-deficient phosphate edia of relatively high sodium content, however, the preferential ab- orption of potassium ions is replaced by that of sodium ions.

3. The presence of potassium ions (0.01 M) in the media has a specific nhancing effect on the assimilation of orthophosphate. This effect is ndependent of the accumulation of potassium ions in the yeast cells. t is as yet undecided whether this influence of potassium ions involves the hysicochemical phase of the entrance of orthophosphate ions into the east cells or its enzymatic transformation to metaphosphate.

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THE HYDROLYSIS OF N-BENZOYL-L-ARGININAMIDE BY CRYSTALLINE TRYPSIN

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(Received for publication, November 26, 1948)

Previous studies of the trypsin-catalyzed hydrolysis of N-benzoyl-L-argininamide to N-benzoyl-L-arginine and ammonia (1, 2) have led to the conclusion that the reaction is first order with respect to both enzyme and substrate and that for any single experiment the rate of hydrolysis may be described by the equation

$$-\frac{ds}{dt} = K'es \tag{I}$$

where e = the total enzyme concentration, s = the substrate concentration, t = the time, and K' = the proteolytic coefficient (3) of the system.

Reinvestigation of the above reaction over a greater range of initial substrate concentrations has now shown that the initial reaction velocity at 25° is essentially independent of the initial substrate concentration for concentrations varying from 0.01 to 0.05 M and that, for these and lesser substrate concentrations, the initial reaction rates obey the Michaelis-Menten (4) equation

$$-\frac{ds}{dt} = \frac{Vs}{K_m + s} \tag{II}$$

within the limits of experimental error. At 25° and pH 7.7 K_m was found to have an apparent value of approximately 0.002 M N-benzoyl-L-argininamide and was shown to be temperature-dependent (Table I). Further it has been observed that for any initial substrate concentration s_0 the reaction rate decreases somewhat more rapidly with time than was expected for a reaction which is truly first order with respect to the substrate concentration and that the reaction products have a marked inhibitory effect upon the reaction rate although ammonium ion alone is without effect.

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* Present address, General Electric Company, Richland, Washington.

† Contribution No. 1251.

Consider the equilibria

$$\begin{array}{ccccccccc} \mathrm{E} & + & \mathrm{BAA} & \overset{K_m}{\longleftrightarrow} & \mathrm{E{\cdot}BAA} & \xrightarrow{k} & \mathrm{E} & + & \mathrm{BA} \quad + \\ (e-p-q) & & (s) & & (p) & & & & \end{array}$$

$$\begin{array}{ccccc} \mathrm{E} & + & \mathrm{BA} & \overset{K_i}{\longleftrightarrow} & \mathrm{E{\cdot}BA} \\ (e-p-q) & & (s_0-s+I) & & (q) \end{array}$$

where BAA = N-benzoyl-L-argininamide, BA = N-benzoyl-L-argin A = ammonia, e = the total enzyme (E) concentration, s = the strate concentration, s_0 = the initial substrate concentration, p = concentration of the enzyme-substrate complex, q = the concentra of the enzyme-hydrolysate complex, I = the initial concentration of hydrolysis products, and $e-p-q$ = the free enzyme concentration.

$$K_m = \frac{s(e-p-q)}{p}$$

$$K_i = \frac{(s_0-s+I)(e-p-q)}{q}$$

From equation (IV)

$$q = \frac{(s_0-s+I)(e-p)}{K_i+(s_0-s+I)}$$

Upon substitution into equation (III)

$$p = \frac{esK_i}{K_m(K_i+s_0-s+I)+sK_i}$$

If $K_i = rK_m$ (r = the ratio of the inhibitor constant to K_m) and − $dt = kp$,

$$-\frac{ds}{dt} = \frac{kes}{K_m+\dfrac{(s_0+I)}{r}-\dfrac{(1-r)s}{r}}$$

Integration gives

$$ket = 2.3\left[K_m+\frac{1}{r}(s_0+I)\right]\log\frac{s_0}{s}-\frac{(1-r)}{r}(s_0-s)$$

With $I = 0$,

$$-\frac{ds}{dt} = \frac{kes}{K_m+\dfrac{s_0}{r}-\dfrac{(1-r)s}{r}}$$

ith $t = 0$,

$$-\frac{ds}{dt} = \frac{kes_0}{K_m + s_0} \tag{VIII}$$

ith $r = 1$,

$$-\frac{ds}{dt} = \frac{kes}{K_m + s_0 + I} \tag{IX}$$

d the reaction would appear to be exactly first order with respect to bstrate.

An equation similar to equation (IX) can be obtained from Klotz's ιear equation (5, 6) for the binding of ions by a protein with n equiva- ιt binding sites per molecule; *i.e.*,

$$\frac{e}{A_e} = \frac{K}{n} \cdot \frac{1}{A} + \frac{1}{n} \tag{X}$$

ιere e = the protein (enzyme) concentration, A = the ion concentra- on, in this case the concentration of the substrate plus the hydrolysis oducts, and A_e = the number of moles of ions bound to protein per ιit volume of solution.

If one takes the reciprocal,

$$A_e = \frac{enA}{K + A}$$

ιd assumes that only the substrate combines with the enzyme,

$$s_e = \frac{ens}{K + s}$$

the rate of reaction is proportional to s_e, the bound substrate concen- ation,

$$-\frac{ds}{dt} = \frac{Kens}{K + s} \tag{XI}$$

quation (XI) has the same form as the Michaelis-Menten equation, but ith a different significance applied to the constants.

If one assumes that the combining sites of the enzyme may be occupied qually well by either the substrate or the hydrolysis products,

$$s_e = \frac{s}{A} A_e = \frac{s}{A} \cdot \frac{enA}{K + A}$$

ιd

$$-\frac{ds}{dt} = \frac{kens}{K + A} \tag{XII}$$

If, in equation (VI), r is less than 1, the hydrolysis products must more firmly bound to the enzyme than is the substrate. In such a ca as the reaction proceeds the rate should decrease more rapidly than wou be predicted on the basis of a first order reaction. Such deviations ha been observed in all experiments in which the initial substrate concentr tions s_0 were relatively high, and at 25° an assumed value for r of (appeared to fit such cases fairly well. However, with low initial substra concentrations the deviation was not observed, most likely because experimental difficulties. The rate constants k, calculated from equ tion (VIII), are given for various initial substrate concentrations, e zyme concentrations, and temperatures in Table I.

TABLE I

Rate Constants at pH 7.7

k = mM minutes^{-1} (mg. of enzyme nitrogen)$^{-1}$.

s_0* (1)	25°; K_m = 0.0021 M		30°; K_m = 0.0033 M	40°; K_m = 0.008
	e = 0.055† (2)	e = 0.034† (3)	e = 0.034† (4)	e = 0.064† (5)
0.050		0.0022		
0.043			0.0065	0.0074
0.030		0.0024	0.0067	0.0076
0.010	0.0023		0.0060	0.0076
0.0087			0.0066	
0.0058			0.0061	0.0073
0.0050	0.0023			
0.0046		0.0018		
0.0040	0.0020			
0.0030	0.0021	0.0021	0.0066	0.0074
0.0020	0.0023			

* mM of N-benzoyl-L-argininamide per ml.

† Mg. of protein nitrogen per ml.

Experimental values for the extent of hydrolysis ($s_0 - s$) at 25° a compared in Table II with those calculated from equation (VI) wi values of $r = 0.5$, 1, and ∞. In general the agreement is better fo = 0.5, although, as pointed out previously at very low initial substra concentrations, a better fit is obtained with $r = 1$.

The effect of the reaction products upon the rate of hydrolysis of benzoyl-L-argininamide by trypsin is probably more clearly shown Table III. It will be seen that 0.05 M ammonium ion causes no demo strable effect. Because of the low solubility of N-benzoyl-L-arginine was not possible to determine its effect upon the system directly and it surprising that it was not precipitated from the reaction mixture. If

TABLE II

Comparison of Experimental and Calculated Results at 25° and pH 7.7

$K_m = 0.0021$ M N-benzoyl-L-argininamide; $k = 0.0022$ mM minutes^{-1} (mg. of enzyme nitrogen)$^{-1}$; $e = 0.055$ mg. of protein N per ml.

s_0 (1)	Time (2)	Hydrolysis (3)	$s_0 - s$, mM per ml. × 10^3			
			Observed (4)	Calculated, $r = 0.5$ (5)	Calculated, $r = 1.0$ (6)	Calculated, $r = \infty$ (7)
mM per ml.	*min.*	*per cent*				
0.010	11.5	11.2	1.12	1.05	1.11	1.13
	20	17.0	1.70	1.73	1.83	1.98
	40	28.5	2.85	2.95	3.31	3.88
0.005	10	16.2	0.81	0.78	0.80	0.83
	20	29.8	1.49	1.35	1.47	1.65
	30	37.0	1.85	1.83	2.02	2.37
	40	45.8	2.29	2.25	2.49	2.97
	50	51.0	2.55	2.57	2.88	
	60	56.8	2.84	2.85	3.20	
0.004	10	16.7	0.67	0.67	0.73	0.78
	20	30.0	1.20	1.22	1.31	1.47
	30	41.8	1.67	1.65	1.79	2.08
	40	48.3	1.93	1.98	2.18	2.55
	50	59.0	2.36	2.26	2.51	
	60	62.8	2.51	2.49	2.77	
0.003	10	20.0	0.60	0.60	0.57	0.67
	20	32.0	0.96	1.05	1.14	1.25
	30	44.7	1.34	1.43	1.53	1.78
	40	59.0	1.77	1.72	1.84	2.16
	50	65.3	1.96	1.92	2.08	
	60	71.0	2.13	2.06	2.27	
0.002	10	26.5	0.53	0.48	0.52	0.57
	20	46.0	0.92	0.83	0.90	1.00
	30	61.5	1.23	1.08	1.18	1.33
	40	69.5	1.39	1.27	1.39	1.57
	50	70.5	1.41	1.42	1.54	1.76
	60	80.5	1.71	1.53	1.68	

had, upon reaching the concentration u, the rate from that time on would be expressed by the equation

$$-\frac{ds}{dt} = \frac{kes}{\left(K_m + \frac{u}{r}\right) + s} \tag{XIII}$$

Consequently, the plot of log s (as ordinate) against time would give a curve which would be concave upward from zero time to the time when the concentration of N-benzoyl-L-arginine became equal to u and concave downward from that time to the end of the reaction. Such an fect was not observed in any of the experiments. In order to circumv the difficulty occasioned by the low solubility of N-benzoyl-L-argininl solution 0.05 M in N-benzoyl-L-argininamide was completely hydroly the enzyme destroyed by heating, and fresh substrate and enzyme ad The rate observed in this solution which was initially 0.042 M in benz L-arginine and ammonium ion was markedly less than that noted in absence of added hydrolysis products and agreement between obse and calculated values was good (Table III). While these data support for the postulate that the hydrolysis products function in an hibitory manner, it should be pointed out that Schwert *et al.* found no

TABLE III

Effect of Reaction Products on Rate of Hydrolysis at 25° and pH 7.7

K_m = 0.0021 M N-benzoyl-L-argininamide; k = 0.0022 mM minutes^{-1} (mg. o zyme nitrogen)$^{-1}$; e = 0.077 mg. of protein nitrogen per ml.; s_0 = 0.050 mM per m

Reaction products added (1)	Time (2)	Hydrolysis (3)	$s_0 - s$, mM per ml. × 10^3		
			Observed (4)	Calculated, $r = 0.5$ (5)	Calcul $r =$ (6
	min.	*per cent*			
None	60	16.8	8.4	8.3	9
	120	29.0	14.5	14.3	16
	270	50.0	25.0	25.0	29
0.05 M NH_4^+	60	16.9	8.5	8.3*	9
	120	29.4	14.7	14.3*	16
0.042 M N-benzoyl-L-arginine, 0.042 M NH_4^+	60	8.3	4.1	3.7†	5
	120	15.5	7.8	6.7†	9
	240	27.3	13.6	12.5†	17

* Assuming no inhibition by ammonium ion.

† Assuming inhibition by N-benzoyl-L-arginine.

dence of inhibitory action by the hydrolysis products in the tryı catalyzed hydrolysis of N-benzoyl-L-arginine methyl ester (7) and it be that both ammonium ion and N-benzoyl-L-arginine are necessar inhibitory action.

EXPERIMENTAL

Reagents—The data presented were obtained with a preparatio crystalline trypsin procured from Armour. Similar results were tained with a Lehn and Fink preparation. Benzoyl-L-argininamide drochloride was prepared according to the procedure of Bergmann, Fr and Pollok (8). The 0.1 M phosphate buffer (pH 7.9) was prepared tassium dihydrogen phosphate and disodium hydrogen phosphate. eagent grade formaldehyde, 40 per cent by volume, was shaken with sic magnesium carbonate, filtered, and the filtrate (pH 8) used for the rmol titrations (9–11). All solutions were prepared with water redis- led from an all-glass apparatus.

Procedure—Generally 5.0 ml. of substrate solution and 1.0 ml. of buf- r solution were pipetted into a 1 × 6 inch test-tube, the tube placed in thermostat, 1.0 ml. of enzyme solution added, the solution shaken, d a 1.0 ml. aliquot immediately withdrawn for a blank determination. fter suitable time intervals, additional 1.0 ml. aliquots were withdrawn. ach aliquot was added immediately to an equal volume of formaldehyde lution contained in a 10 ml. beaker, and the beaker placed in the depres- on of a rubber stopper which was mounted on the shaft of an inverted r-driven stirrer in such a way as to permit rotation of the beaker during e titration. The glass and reference electrodes immersed in the solu- on facilitated stirring. The solution was titrated with standard 0.01 M dium hydroxide in a semiautomatic burette graduated in 0.01 ml. and quipped with a capillary tip of sufficient length to permit introduction the reagent beneath the surface of the solution being titrated; thus the isadvantages inherent in drop-wise transfer are avoided. The end- oint of the titration (pH 8.1) was determined with the aid of a Beckman odel G pH meter equipped with electrodes specially constructed for use the limited space available. The glass electrode was of the type in- ended for use in alkaline solutions. After every titration the electrodes ere washed with distilled water and checked against a standard buffer lution. The solutions were invariably titrated immediately after the liquot had been added to the formaldehyde solution. Blank runs made ith solutions containing no enzyme indicated that the substrate was ot hydrolyzed in the absence of the enzyme under the conditions em- loyed.

SUMMARY

A reinvestigation of the kinetics of hydrolysis of N-benzoyl-L-arginina- ide by crystalline trypsin has led to the conclusion that the hydrolysis roducts enter into the over-all reaction as inhibitors.

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THE CONFIGURATION OF THE ALLOPREGNANETRIOL-3,16,20 OF THE URINE OF PREGNANT MARES*

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In 1933 Smith, Hughes, Marrian, and Haslewood (1) reported the isolation of a new compound from the urine of pregnant mares. The substance was studied extensively in the laboratories of Marrian (2, 3) and of Marker (4–7) and was shown to be a saturated triol of the composition $C_{21}H_{36}O_3$. Marker and his collaborators, who reported the conversion of this compound to allopregnane, to allopregnanedione-3,20, to Δ^{16}-allopregnenedione-3,20, and to 3-ketoetioallobilianic acid, proposed the structure of an allopregnanetriol-3(α),16,20 for the new triol. Since these reactions involved carbon atoms 3, 16, 17,[1] and 20, no inferences can be drawn about the configurations at these asymmetric centers. Nevertheless, since the compound failed to precipitate with digitonin, it was assigned the α configuration at carbon atom 3 (5, 3, 6). Normal arrangement at C-17 was implied by designating it as a derivative of allopregnane.

The present study of this compound was undertaken when it appeared that the reduction product[2] of a triol isolated from human urine (8) might be an allopregnanetriol-3(β),16,20 differing from Marrian's compound only in the configuration at C-3. This supposition has not been borne out, since our attempts to synthesize such a compound from the triol from mare urine brought out the fact that Marrian's compound, which is not identical with our reduction product, already possesses the β configuration

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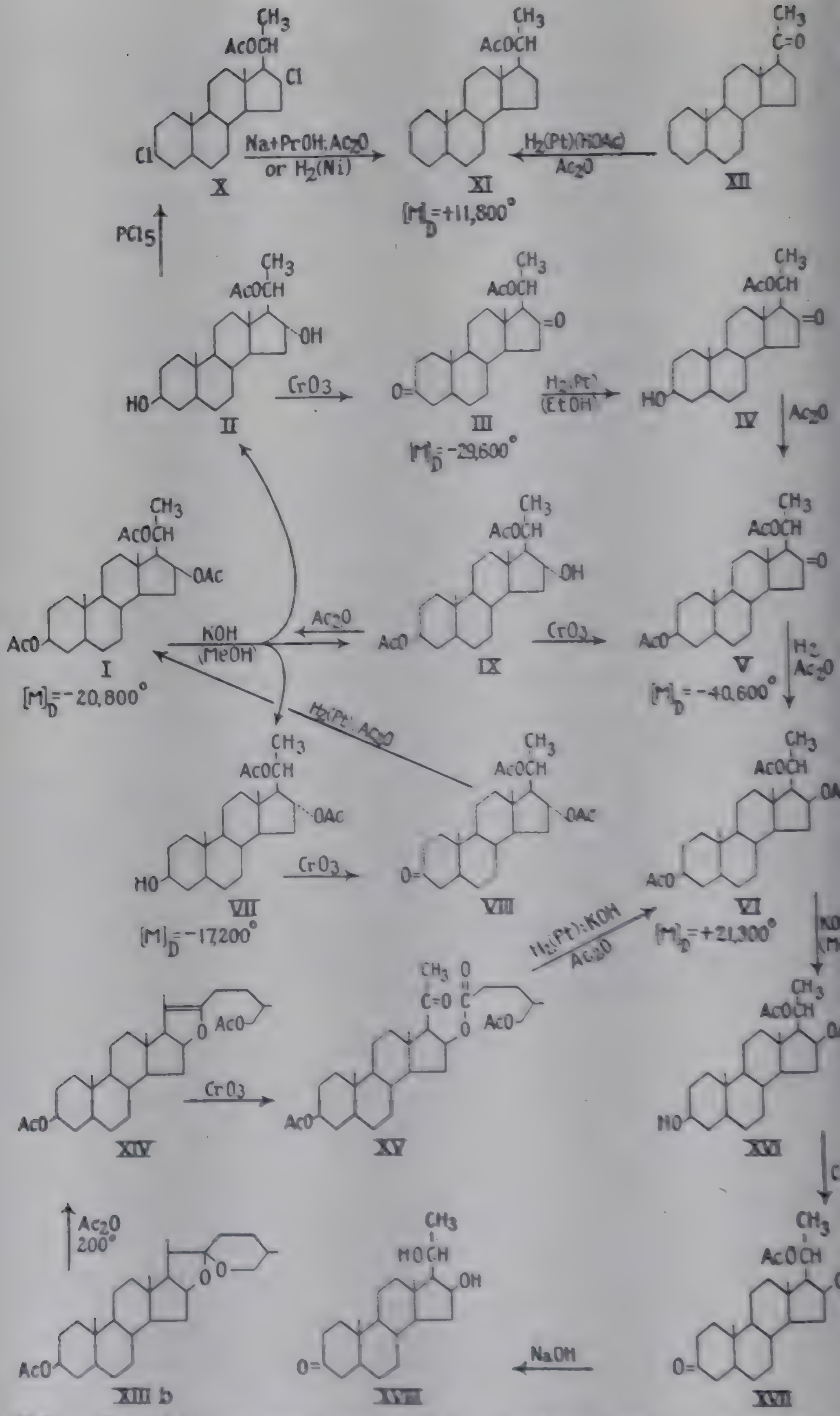
[1] Since Marker's explanation (5) of the formation of allopregnanedione-3,20 is no longer tenable (6), it would appear that Δ^{16}-allopregnenedione-3,20 is an intermediate in this reaction. A Δ^{17-20}-unsaturated intermediate is strongly indicated (6) in the formation of allopregnane by the Clemmensen reduction of 20-acetoxyallopregnanedione-3,16. Finally, the hydrocarbon first identified as pregnane (5) and subsequently as allopregnane (6), which was obtained in small yield from the triol when it was treated with phosphorus pentachloride and reduced, was reported to be formed via an olefinic intermediate. All of these transformations, therefore, seem to involve C-17.

[2] Unpublished experiments from this laboratory.


X
Na+PrOH; Ac2O or H2(Ni)
XI
[M]D = +11,800°
H2(Pt)(HOAc) Ac2O
XII
PCl5
II
CrO3
III
[M]D = -29,600°
H2(Pt) (EtOH)
IV
Ac2O
I
[M]D = -20,800°
KOH (MeOH)
Ac2O
IX
CrO3
V
[M]D = -40,600°
H2 Ac2O
H2(Pt) Ac2O
VII
[M]D = -17,200°
CrO3
VIII
VI
[M]D = +21,300°
H2(Pt); KOH Ac2O
XIV
CrO3
XV
XVI
Ac2O 200°
XIII b
XVIII
NaOH
XVII

at C-3. In addition, evidence has been obtained pertaining to figurations at the asymmetric centers 16, 17, and 20 which we completes the structure proof of this substance.

dell and Marrian showed that the triacetate (I) of the compound from
;nant mare urine when treated with 0.8 M equivalent of potassium hy-
ide in methanol loses two acetyl groups to form a triol monoacetate
. This was oxidized to the acetoxy diketone (III) (3) which proved
e very labile to both acid and alkali (6). Under these conditions
ic acid was eliminated and an olefinic diketone formed which in turn
converted to a saturated diketone (6). As the latter melted at a
r temperature than did either allopregnanedione-3,20 or 17-isoallo-
nanedione-3,20, Marker and Wittle inferred that the monoacetates
nd III contain their acetoxy groups in position 20. We have obtained
ct evidence that this conclusion is correct. Treatment of the triol
oacetate (II) with phosphorus pentachloride furnished the acetoxy di-
ride (X) which on reduction with either sodium and propanol and re-
ylation or with hydrogen in the presence of a Raney nickel catalyst
afforded in good yield the acetate of a monohydric alcohol, $C_{21}H_{36}O$.
s substance proved to be identical with a specimen of allopregnanol-
3) acetate[3] (XI) (11) that had been obtained from allopregnanone-20
I). This sequence of reactions proves that the monoacetates II and
are esterified in position 20, that this substituent possesses the β con-
ration, and that the triol and its acetates are normal with respect to
spatial arrangement of the side chain which is therefore cis (13) to
angular methyl groups.

Vhen an alcoholic solution of the diketone III was shaken with hydro-
in the presence of platinum, the reaction ceased after only one keto
up had been reduced. The main reaction product (IV), which precipi-
ed with digitonin, was converted to the diacetate (V). The presence
a carbonyl group in this compound was demonstrated conclusively by
mination of its absorption in the ultraviolet region, which showed a
ximum at 297 mμ (Fig. 1). It follows from the structure of the dike-
e III that the carbonyl group in compounds IV and V must be located
either C-3 or C-16. Resistance towards catalytic hydrogenation has
n observed for certain 16-ketones such as tigogenoic acid (14) (3(β)-

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The configurations of the substituents at C-20 are designated in accordance
h Marker who proposed the urinary pregnanediol-3,20 as the reference com-
nd for the 20(α) configuration. Our reasons for believing that urinary allo-
gnanediol-3(β),20 has the α configuration at C-20 have been stated previously
Allopregnanol-20(α)-one-3 has been reduced to this diol (10) and by a modi-
Clemmensen reaction to allopregnanol-20(α) (11). The second allopregnanol-
(11) obtained by oxidation and subsequent reduction of the α form should,
refore, be the 20(β) isomer. The configurations of the nuclear substituents are
ed α and β in accordance with the suggestions of Reichstein and Shoppee (12).
e the attachment of the side chain in the natural bile acids has recently been
nd (13) to be cis to the angular methyl groups and is accordingly designated as
he indices used in this paper do not always agree with those of the references
d.

hydroxy-16,22-dioxocholestanoic acid-27 (15)) but not for 3-ketoster
The infra-red absorption spectrum of compound V (Fig. 2, Curve C) i
cated the presence of a carbonyl peak which was not well resolved f
that caused by the two acyl groups but showed no maximum in the re
where ketones in position 3 have been reported to absorb (5.82 μ) (
Finally, when the diacetate V was tested in the von Bittó-Zimmerm
reaction, it gave a spectrum wholly different from those obtained
3-ketosteroids ((17) and unpublished data) (Fig. 3). It is conclu
therefore, that compound V is a 3,20-diacetoxy-16-ketone. Its inert k
group could be reduced, however, with platinum in acetic acid or wit

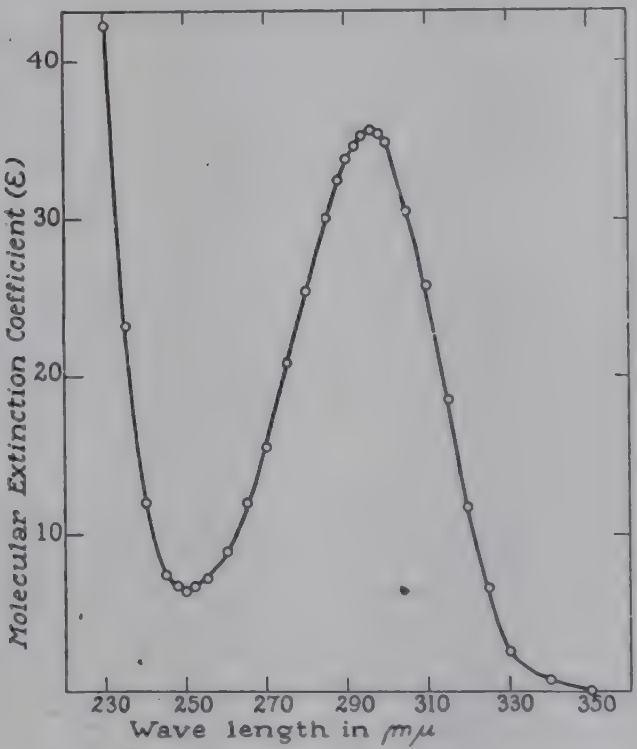


FIG. 1. Ultraviolet absorption spectrum of allopregnanediol-3(β),20(β)-on
diacetate (V) in 95 per cent ethanol.

Raney nickel catalyst (18) in alcohol or far less satisfactorily with
minum isopropylate. The reaction product which was isolated after ace
ation was the same triol triacetate (VI) in each instance. As its melt
point was close to those of two allopregnanetriol-3(β),16,20 triacet
that had been prepared by Marker and his collaborators (19–21) f
tigogenin (XIIIa), we repeated their preparations (XIIIb → XIV →
→ VI and XIIIb → XIX (22) → XX). The isomer obtained from p
dotigogenin diacetate (XIV) showed the same melting point, rotat
and infra-red spectrum (Fig. 2) as the triacetate (VI) prepared f
compound I. The identity of both preparations of compound VI
confirmed by mixed melting point determinations on the triacetates

the free triols. It follows that the triacetate from pseudotigogenin diacetate, like compound XI, has its 20-acetoxy group in the β configuration. Marker drew the same conclusion from the steric course of the reduction of the 20-keto group of compound XV (23), but the reliability of this criterion has been questioned (24). Since tigogenin is a 3(β)-hydroxysteroid (19), the same configuration must be assigned to compounds IV, V, and VI. The steps used in the conversion of triacetate I to triacetate VI admit the possibility of inversion at C-3 and at C-16. Inversion at C-17, while conceivable in 16-ketosteroids, is regarded as highly improbable. Moreover, since both starting materials (I and XIIIb) used in the synthesis of compound VI possess the normal β configuration of the side chain, epimerization would have had to occur on both routes. The second of these proceeds via pseudotigogenin diacetate, which, as set forth below, should still possess β configuration at C-17. The succeeding steps entail chromic acid oxidation to a 20-ketosteroid and its catalytic hydrogenation. In 20-ketosteroids with normal trans fusion of Rings C and D the β configuration of the side chain has been found to be more stable (25–30)

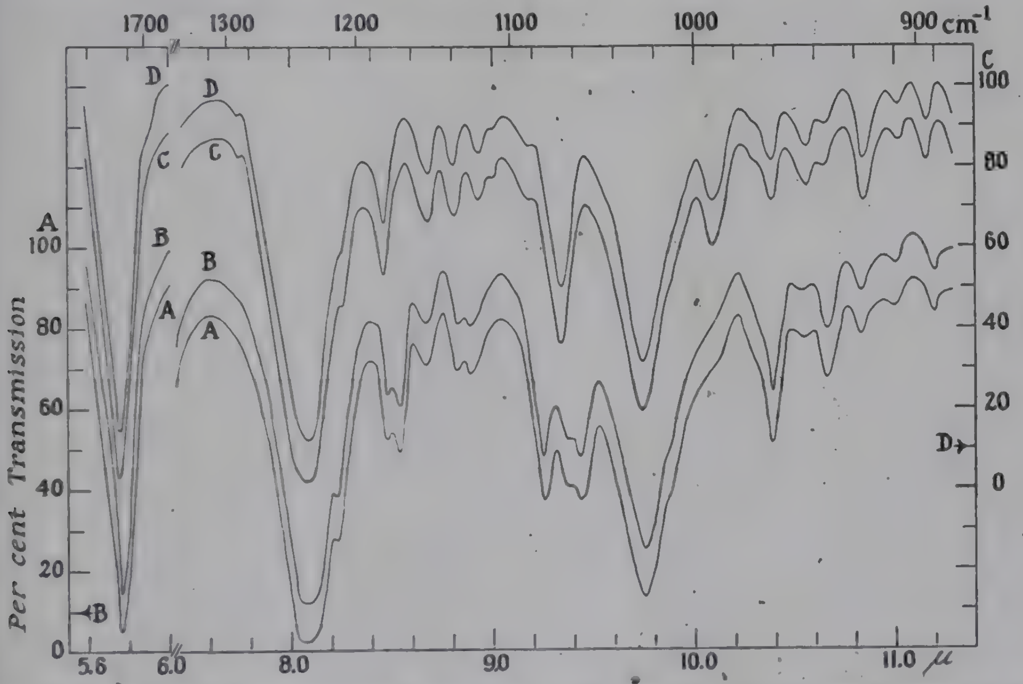


FIG. 2. Infra-red transmission spectra. Curve A, allopregnanetriol-3(β),16-(β),20(β) triacetate (VI) from compound V; Curve B, allopregnanetriol-3(β),16-(β),20(β) triacetate (VI) from compound XIV; Curve C, allopregnanediol-3(β),20-(β)-one-16 diacetate (V) from compound IV; Curve D, allopregnanediol-3(β),20-(β)-one-16 diacetate (V) from compound IX. Curve A is drawn with reference to the ordinate at the left, Curve C to that at the right, while Curves B and D are displaced by +10 from Scales A and C, respectively (the zero points are indicated by Arrows B and D). The abscissa is linear with respect to wave-length. Concentration, 1 mg. in 0.1 cc. of carbon disulfide; cell spacer, 0.8 mm.

than the α form and its inversion to the α configuration under the co
tions of these reactions has never been described.

Evidence that the conversion of compound I to VI alters the con
ration at C-16 but not at C-3 was obtained by a study of two diacet

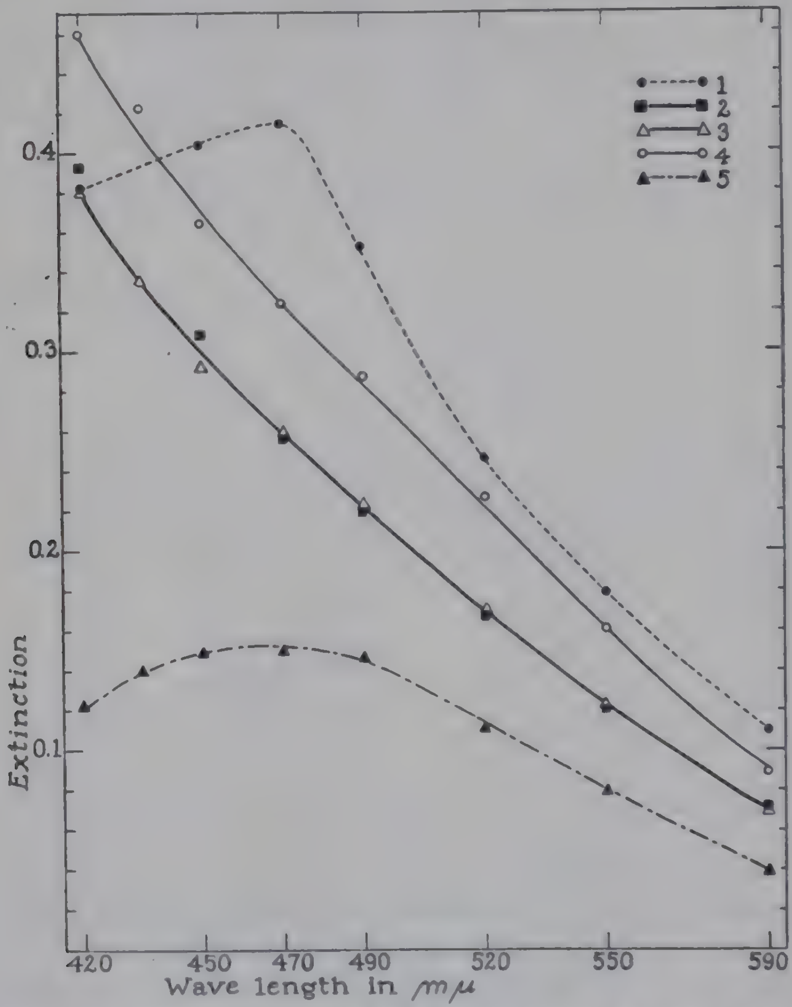
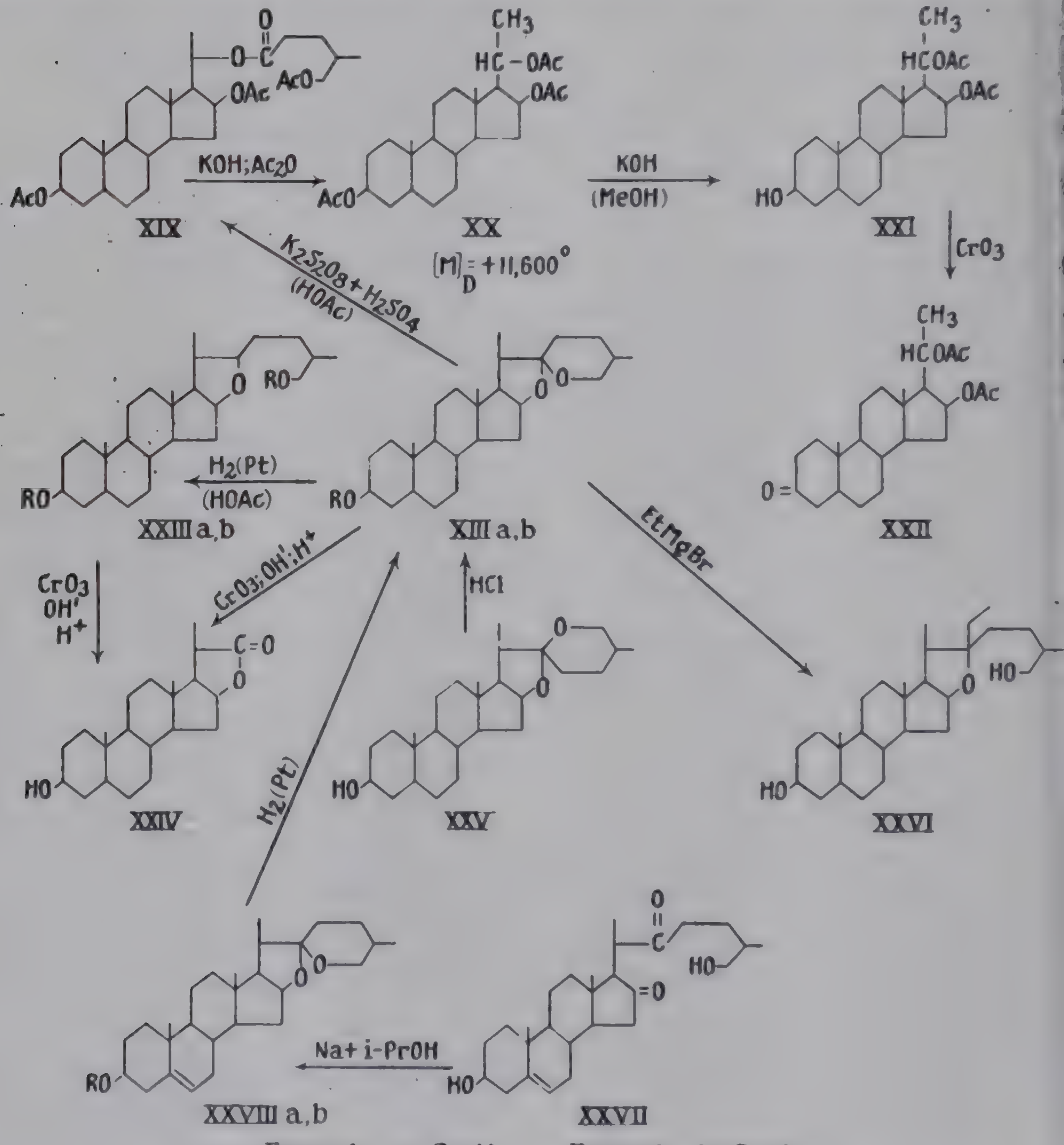


FIG. 3. Absorption measurements of pigments formed with alkaline *m*-din
benzene. Curve 1, allopregnanediol-3(β),20(β)-one-16 diacetate (V); Curv
allopregnanediol-16(α),20(β)-one-3 diacetate (VIII) (crude oxidation prod
Curve 3, allopregnanediol-16(β),20(α)-one-3 diacetate (XXII); Curve 4, allo
nanediol-16(β),20(β)-one-3 diacetate (XVII) (crude oxidation product); Curv
allopregnanone-20 (XII). Concentrations for Curves 1 to 4, 100 γ per 0.2 c
alcohol; for Curve 5, 75.6 γ per 0.2 cc.; reaction time, 105 minutes. Condi
as previously reported (17).

of Marrian's triol. Previous attempts directed at the preparation of
products by treatment of a methanolic solution of the triacetate wi
molar equivalent or less of potassium hydroxide (5, 3) have led only t
isolation of the monoacetate II. The reports (6, 3) indicated, how
and our findings confirmed that the resulting reaction mixtures still
tained an appreciable portion of the alkali added, suggesting that

hain process is an alkali-catalyzed methanolysis rather than a saponificaion (31–33). While the amount of base, therefore, does not effectively mit the extent of the reaction, it seemed probable that intermediates ould be obtained if the alkali concentration or the reaction time or temerature was decreased. When the reaction time was shortened to 16 ours, a complex mixture resulted which yielded two diacetates (VII and X) in addition to the monoacetate II and unchanged starting material. he higher melting diacetate (VII) which was obtained in a far higher ield was oxidized with chromic acid. The reaction product (VIII) failed o crystallize but showed in the von Bittó-Zimmermann reaction the color hanges and absorption spectrum characteristic of a 3-ketosteroid (Fig. 3). ompound VII, therefore, is the 16,20-diacetate of Marrian's triol. Cataytic reduction of ketone VIII in either a neutral or an acidic medium ielded after acetylation compound I as the main product. The Auwers-kita rule, therefore, cannot be applied and the steric course of the reducion does not aid in determining the configuration of the carbon-oxygen inkage at C-3 in compound I. The acetylation of the hydroxyl group in ompound VII is accompanied by a decrease in molecular rotation equal to 3600°, which in the allo series is characteristic of β-3-hydroxysteroids but not of their 3-epimers, which show an increase of about 2400° during this reaction (34). Direct proof that compound I is a β-3-acetoxysteroid was obtained by the oxidation of the second diacetate (IX). The reaction product was identified as compound V by its melting point and mixed melting point as well as by its absorption spectrum in the finger-print region of infra-red radiation (Fig. 2). Since compound IX was obtained only in very small amounts, it seemed important to show that it was derived from compound I rather than from an accompanying impurity. The reacetylation of the diacetate IX to the triacetate I leaves no doubt, however, that compound IX is a derivative of Marrian's triol. It must be concluded, therefore, that the 3-acetoxy group in triacetate I is β-oriented, as it is in compound V.

The configurations of the 16-acetoxy groups in these compounds were deduced from considerations of the known reactions of the sapogenins as well as from a comparison of the rates of solvolysis of the 16-acetates. The condensation of two five-membered rings, as is present in the sapogenin molecule, admits the possibility of both cis and trans fusion. However, while the cis arrangement possesses about normal bond angles, the trans compounds are definitely under strain (35–39), and if they contain reactive groups which would permit its release they may be expected to be unstable. It is apparent that the furanoid ring of the sapogenins is not only readily formed but also is stable under conditions which lead to the opening of the pyranoid ring. An example for the ready formation of the spiroketal ring system present in tigogenin (XIIIa) is the conversio kryptogenin (XXVII) to diosgenin (XXVIIIa) by reduction with sod and isopropanol (40). Neotigogenin (XXV) on treatment with hy chloric acid in ethanol is epimerized at C-22 to yield tigogenin (XI (41). While the mechanism of this reaction has not yet been elucida it must involve opening of at least one of the rings. If the furanoid


XIX
KOH;Ac$_2$O
XX
[M]$_D$ = +11,600°
KOH (MeOH)
XXI
CrO$_3$
XXII
K$_2$S$_2$O$_8$+H$_2$SO$_4$ (HOAc)
H$_2$(Pt) (HOAc)
XXIII a,b
XIII a,b
EtMgBr
CrO$_3$ OH' H$^+$
CrO$_3$;OH';H$^+$
HCl
XXIV
H$_2$(Pt)
XXV
XXVI
Na+ i-PrOH
XXVIII a,b
XXVII

Formula a, R = H Formula b, R = Ac

in tigogenin were under strain, it could hardly be expected to be sta under the conditions of the reaction. Treatment with Grignard reag (21) opens the pyranoid but not the furanoid ring (XIIIa → XXVI), the same has been shown for catalytic hydrogenation in an acid medi (XIIIa → XXIIIa) (42). Evidence that the furanoid ring remained changed during the reduction can be seen in the oxidation of dihyd tigogenin diacetate (XXIIIb) to the same lactone (XXIV) (15) that is orded by the oxidation of tigogenin acetate (XIIIb) (43). The lactone an be hydrolyzed to the sodium salt of the corresponding hydroxy acid but acidification suffices to reform the lactone. An equally ready actonization was observed by Hueckel and Gelmroth with *cis*-2-hydroxycyclopentaneacetic acid (44, 38). In contrast the trans acid remained argely unchanged, even under forcing conditions (44). The neutral products obtained from it by various drastic procedures consisted mainly or entirely of the cis lactone (44, 45). Obviously the trans lactone if it was formed at all can be obtained only with great difficulty. It is concluded, therefore, that compound XXIV is likewise a cis lactone. All of these observations indicate strongly that the carbon-oxygen linkage at C-16 in tigogenin is cis to the carbon-carbon bond at C-17. Since the latter has the normal β configuration, as was shown by the conversion of diosgenin (XXVIIIa) to cholesterol (46), the same assignment (β) is made for the carbon-oxygen bond at C-16. It would appear that both linkages remain unaffected when tigogenin is converted to the two isomeric triacetates (VI and XX). In particular the reactions involving the oxygen atom at position 16 seem to be confined to the formation and hydrolysis of esters and to the acetolysis of a ketal and should, therefore, not affect the bond with C-16. While the presence of a double bond in pseudotigogenin diacetate could lead to epimerization at C-17, such a reaction is most unlikely[4] when the starting compound already possesses the stable cis configuration. Furthermore, if the linkages at C-16 and C-17 remain unchanged, Marker's contention (23) should be correct that the triacetates VI and XX are epimeric at C-20. Although the vicinal effect of a 16-acetoxy group or the disturbances due to solvent variation may not be negligible, it seems significant that the difference in molecular rotation between these triacetates is close to that reported for the pair of 20-epimeric pregnanediol-3(α),20 diacetates.[5] The conclusion, therefore, seems warranted that triacetate VI is to be designated as allopregnanetriol-3(β),16(β),20(β) triacetate, its 20-epimer (XX) as allopregnanetriol-3(β),16(β),20(α) triacetate, and its 16-epimer (I), the triacetate from mare urine, as a pregnanetriol-3(β),16(α),20(β) triacetate.

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[4] A serious objection to this contention could be based on Marker's suggestion that the cyclic structure of pseudotigogenin (as given in the diacetate XIV) is tautomeric with that of an open chain 22-hydroxy-$\Delta^{16,20-22}$-diene (20), since such a shift could lead to inversion at both C-16 and C-17. It should be noted, therefore, that this concept and its experimental support have been withdrawn (47). The ready reconversion of pseudotigogenin to tigogenin (48) can be looked upon as another indication that both compounds have identical configurations at C-16 and C-17.

[5] Meystre and Miescher (49) report for pregnanediol-3(α),20(α) diacetate $[\alpha]_D = +26° \pm 4°$ in benzene and for pregnanediol-3(α),20(β) diacetate, $[\alpha]_D = +45° \pm 4°$ in chloroform. Hence, $\Delta[M]_D = 7700°$, while the difference in the molecular rotations (in 95 per cent ethanol) of the triacetates XX and VI equals 9700°.

As a further test for the correctness of these assignments the rela rates of the removal of the 16-acetyl groups with methanolic potass hydroxide were studied under standardized conditions. In order avoid secondary changes the reaction mixtures were examined with concentrating the methanolic solution and without recourse to chroma graphic analysis (50). The main reaction product in every case obtained in a yield higher than 50 per cent. While compound I converted to the 20-monoacetate (II), the triacetates VI and XX yiel diacetates (XVI and XXI). Both diacetates were unesterified in posit 3, since chromic acid oxidation furnished products (XVII and XXII) w the chromogenic properties of 3-ketosteroids (Fig. 3). This was c firmed in the case of compound XVII by alkaline hydrolysis, which yiel a ketodiol (XVIII) rather than a monohydroxylated α,β-unsaturated tone which would have resulted if the carbonyl group were either in p tion 16 or 20 (6, 20). It follows, therefore, that the 16-acetoxy grou more reactive in the α than in the β configuration. The only substitu which seems large enough and close enough to hinder this reaction is side chain if it is cis to the reacting group. As the side chains in all th triacetates have the β configuration, the results of the solvolysis react are in accord with the proposed structures.

Reference should be made to two other urinary steroids containing hydroxyl groups, the configurations of which have been elucidated. H man and Lott (51) obtained very good evidence that the 17-hydro groups in estriol and in Δ^5-androstenetriol-3,16,17 possess the same ori tation as the one in testosterone. Since the 16-hydroxyl groups w found to be trans to those at C-17, they were designated as β, an assi ment which was in full accord with prevailing nomenclature. Recent e dence indicates, however, that the 17-hydroxyl group of the refere compound, testosterone, may not be trans but cis to the angular met group at C-13 (52). If this is correct, the three 16-hydroxylated tri that have been isolated from urine (estriol, androstenetriol, and allopr nanetriol) are all 16(α)-hydroxysteroids. The β configuration has b proposed for the 16-hydroxyl group of gitoxigenin by Shoppee (24) a tentatively by Meyer (53). It seems premature, however, to correl this assignment with those made in the present report.

The demonstration that Marrian's triol is a β-3-hydroxysteroid nece tates a reinterpretation of Marker's unsuccessful attempts to epimerize hydroxyl group at C-3. When the triol was heated with sodium in lene, only a small amount of material precipitable with digitonin formed (5). This was ascribed to the insolubility of the triol in xyle since treatment with sodium in amyl alcohol furnished large amounts of insoluble digitonides (6). These upon splitting did not yield a triol but a condensation product, $C_{26}H_{46}O_2$. A negative result in the first experiment is to be expected, since allopregnanetriol-3(β),16(α),20(β) does not precipitate with digitonin, while the outcome of the second must be ascribed not to inversion at C-3 but to other reactions, such as condensation with amyl alcohol. The structural factors which prevent the precipitation of allopregnanetriol with digitonin have not been elucidated. While Marrian's triol is quite insoluble in dilute alcohol and can be tested only in rather low concentration, other related compounds that are more soluble also failed to precipitate. Among the three β-3-hydroxy-16,20-diacetates, compounds VII, XVI, and XXI, the 20(α) compound, substance XXI, was the only one to yield a precipitate. The negative results with the two other diacetates cannot be ascribed to the β configuration of the 20-acetoxy group, since compound IV precipitated promptly with digitonin.

EXPERIMENTAL[6]

Isolation of Allopregnanetriol-3(β),16(α),20(β) As Triacetate (*I*)—Since we were unable to isolate allopregnanetriol from the material at our disposal according to the procedures given in the literature (2, 4), we are describing the modification used here. The neutral fraction of the urine of pregnant mares was received from E. R. Squibb and Sons as a dark oil of low viscosity. 500 cc. of this material (485 gm.) were mixed with a solution of 80 gm. of potassium hydroxide in 640 cc. of 95 per cent ethanol, heated under a reflux for 1.5 hours, and after cooling distributed between 2.5 liters of ether, 2 liters of water, and 470 gm. of sodium chloride. The aqueous phase was reextracted with 1000 cc. and 500 cc. of ether. The ether layers were combined and washed with 1400 cc. of water and then alternately with 800 cc. portions of 4 per cent sodium hydroxide solution and water until most of the alkali-soluble pigments had been removed. The sodium hydroxide which was added as a 10 per cent solution caused the formation of a precipitate which was separated before dilution and equilibration. The removal of this material prevented the formation of troublesome emulsions in the succeeding washings with water. The organic phase was washed with 800 cc. of 4 per cent hydrochloric acid and with water until neutral and evaporated. The residue was steam-distilled until about 9 liters of distillate had been collected. The non-volatile material was extracted with 3 liters of ether, and the ether solution was washed with 4 per cent sodium hydroxide (700 cc.), with water (700 cc.),

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[6] All melting points reported are corrected. Rotations were determined in 95 per cent ethanol except when noted otherwise. Samples were dried for analysis and rotation at 80° *in vacuo*, except compound XVIII which was dried at 110°.

Allopregnanetriol-3(β),16(α),20(β) 3,20-Diacetate (IX)—Fraction S (6 mg.) was dissolved in 3 cc. of a 2:1 mixture of petroleum ether and b zene and passed through a column (69 × 6 mm.) of alumina. The seco eluate (14.6 mg.) was obtained with 10 cc. of the same solvent mixtu It was recrystallized repeatedly from dilute methanol and from petrole ether and yielded 10.0 mg. of fine needles melting at 115–116.5°.

Analysis (*F. S.*)—(Sample 1.1 mg.) $C_{25}H_{40}O_5$. Calculated. C 71.39, H 9.59
Found. " 71.44, " 10.81

Although the low melting points of several fractions indicated the pr ence of additional amounts of this diacetate, attempts to isolate it by crystallization or rechromatographing were unsuccessful. The analy could, therefore, not be repeated.[7]

1.0 mg. of this diacetate was acetylated at room temperature with a 2 mixture of pyridine and acetic anhydride (17 hours). The reaction pro uct after two recrystallizations from dilute methanol gave 0.9 mg. platelets melting at 166.5–169.5° and at 169.5–171° on admixture w a sample of pure triacetate (I).

48 Hour Solvolysis of Allopregnanetriol-3(β),16(α),20(β) Triacetate (20-Monoacetate (II)—A solution of 334.5 mg. of allopregnanetriol-3(β 16(α),20(β) triacetate (I) and of an equimolar amount of potassium h droxide in methanol (total volume 117 cc.; concentration 0.00618 m per liter) was kept at 20.0° ± 0.1° for 48 hours, and then distributed b tween 350 cc. of water and 500 cc. of ether. The ether phase was wash until neutral and yielded on evaporation 276.6 mg. of residue (avera molecular weight found 382; calculated for $C_{23}H_{38}O_4$, 378.5). 71.7 mg. this material were extracted with benzene as described above; the mainder upon recrystallization from 95 per cent ethanol yielded 11 mg. of the monoacetate melting at 227–230° and 67.6 mg. of a less pu product melting at 221–228°. With the inclusion of this material yield was 65 per cent. The melting points reported in the literature this compound are 222–224° (3) and 233–235° (6).

Allopregnanol-20(β)-dione-3,16 Acetate (III)—This compound was p pared from allopregnanetriol-3(β),16(α),20(β) 20-monoacetate (II) ess tially by the procedures given in the literature (3, 6) (3 hours oxidat at 25°) and recrystallized from dilute alcohol. It melted at 191–193

[7] *Addition to proof (February 14, 1949).* Through the generosity of Dr. L Engel we received a large sample of Marrian's triol which enabled us to repeat preparation of compound IX. Analysis (E. H.), found, C 71.00, H 9.38. Altho the new H value is satisfactory, some reservation should still be expressed about purity of this product which may not be entirely free of isomer VII. The prese of such an impurity, however, would in no way invalidate the conclusions drawn f the conversion to ketone V.

Rotation, $[\alpha]_D^{27} = -79°$ ($c = 0.5$) (sample dried at room temperature). In the dinitrobenzene reaction it gave an intense purple color which changed to brown within about 8 minutes. Measurements of the extinction of the final color product showed unusually large variations and are, therefore, not reported.

Reduction of Allopregnanol-20(β)-dione-3,16 Acetate (III) to Allopregnanediol-3(β),20(β)-one-16 20-Acetate (IV)—15.9 mg. of platinum oxide (according to Adams and Shriner) were suspended in 4 cc. of 95 per cent ethanol and reduced with hydrogen. A solution of 82 mg. of compound III in 10 cc. of the same solvent was added and shaking continued until the hydrogen uptake ceased (2 hours, apparent hydrogen uptake 1.4 moles equivalent). The catalyst was removed by centrifuging, the reduction product was separated with digitonin (319 mg.) in a medium of 32 cc. of 80 per cent ethanol into a precipitable and a non-precipitable fraction, which were worked up in the usual manner (54). The precipitable material (63.9 mg.) upon recrystallization from mixtures of benzene and petroleum ether (4:10) yielded 60.6 mg. of fine needles melting at 190–193°. Further recrystallization raised the melting point to 192.5–194°.

Analysis (*J. A.*)—$C_{23}H_{36}O_4$. Calculated, C 73.36, H 9.64; found, C 73.74, H 9.19

Allopregnanediol-3(β),20(β)-one-16 Diacetate (V) from 20-Monoacetate (IV)—51.8 mg. of allopregnanediol-3(β),20(β)-one-16 20-monoacetate (IV) were acetylated with 1 cc. of acetic anhydride and 2 cc. of pyridine at room temperature (16 hours). The reaction product crystallized from methanol in long needles which melted at 184–186° (43.8 mg.). The mother liquors furnished 9 mg. of the diacetate melting at 182.5–185°.

Analysis (*J. A.*)—$C_{25}H_{38}O_5$. Calculated, C 71.74, H 9.15; found, C 71.68, H 8.88
Rotation, $[\alpha]_D^{20} = -97°$ ($c = 0.4$)

The absorption spectrum of this compound in the ultraviolet region is given in Fig. 1, in the infra-red in Fig. 2 (Curve C). In the dinitrobenzene reaction it yielded a bronze-colored solution. Absorption data on the final product are given in Fig. 3 (Curve 1).

Reduction of Allopregnanediol-3(β),20(β)-one-16 Diacetate (V); (a) With Platinum—18.7 mg. of compound V, 16 mg. of platinum oxide, and 6 cc. of glacial acetic acid were shaken at room temperature in an atmosphere of hydrogen until no more gas was taken up. The reaction product was freed of catalyst and solvent and acetylated at room temperature with 1 cc. of acetic anhydride in 2 cc. of pyridine (38 hours). The crude triacetate (19.8 mg.) was recrystallized repeatedly from methanol and from petroleum ether. 11.5 mg. of heavy crystals with numerous facets were obtained which melted at 165–165.5° and at 164–165.5° on admixture with a sample of allopregnanetriol-3(β),16(β),20(β) triacetate (VI) (m 164–165°) prepared from pseudotigogenin diacetate (XIV). Admixt with the triacetate of allopregnanetriol-3(β),16(β),20(α) (XX) marke depressed the melting point.

Analysis (*J. A.*)—$C_{27}H_{42}O_6$. Calculated, C 70.10, H 9.15; found, C 70.46, H 9. Rotation, $[\alpha]_D^{30} = +46°$ (c = 0.5)

(*b*) *With Nickel*—A solution of 7.4 mg. of compound V in 4 cc. of etl nol and a suspension of 36 mg. of a W-4 nickel catalyst (18) in 0.06 cc. the same solvent were shaken at room temperature in an atmosphe of hydrogen at normal pressure for 150 minutes; no uptake of gas cou be observed during this period. The reaction product was acetylated room temperature (16 hours) and then recrystallized as described abo The melting point (165–166°) of the triacetate (4.3 mg.) was not depress by admixture with the preparation obtained by method (*a*). Transm sion measurements in the infra-red region are reported in Fig. 2, Curve

(*c*) *Other Procedures*—Unsuccessful attempts were made to reduce co pound V with sodium amalgam in alcoholic acetic acid (55) or catalytical (Pt) in an alcoholic solution at 75° at a hydrogen pressure of 200 pound Reduction of 9.8 mg. of compound V with 70 mg. of aluminum isopr pylate in boiling isopropanol (5.0 cc.) followed by acetylation and crystallization furnished 1.0 mg. of a product which melted at 160–16 and at 163–165.5° on admixture with a sample of pure compound VI.

Allopregnanetriol-3(β),16(β),20(β)—4.3 mg. of triacetate VI prepared procedure (*b*), 40 mg. of sodium hydroxide, and 5 cc. of 80 per cent eth nol, were heated under a reflux for 70 minutes. The resulting triol (mg.) was obtained by ether extraction and recrystallized from acetone a twice from methanol. Its melting point was found to be rather variab the highest observed was 274–284° with decomposition. It was not d pressed by admixture with a specimen prepared from pseudotigogeni which melted at 273–284° with decomposition. The compound has be reported to melt at 286–288°, 285–288°, and 285–287° (20, 23).

Oxidation of Allopregnanetriol-3(β),16(α),20(β) 16,20-Diacetate (VII) 16.7 mg. of compound VII were dissolved in 1 cc. of acetic acid and o dized with a solution of 4.0 mg. of chromium trioxide in 0.33 cc. of 90 p cent acetic acid for 3 hours at 25° and then treated with 0.5 cc. of meth nol. The reaction mixture was distributed between ether and wat The ether layer was washed with water, repeatedly with sodium carbona solution, and with water, and yielded on evaporation 14.5 mg. of a colo less oil which could not be induced to crystallize. Spectrographic mea urements on the pigment formed from such material with alkaline di trobenzene are given in Fig. 3, Curve 2. Separation with Reagent of Girard and Sandulesco yielded 13.2 mg. of material in the ketonic and 1.1 mg. in the non-ketonic fraction.

Oxidation of Allopregnanetriol-3(β),16(α),20(β) 3,20-Diacetate (IX)—A solution of 6.9 mg. of diacetate IX in 1 cc. of acetic acid was mixed with a solution of 1.7 mg. of chromium trioxide in 0.12 cc. of 90 per cent acetic acid. The mixture was kept at 25° for 3 hours and worked up as described above. The neutral fraction was recrystallized three times from methanol and yielded 2.6 mg. of long needles which melted at 183.5–185.5° and at 184.5–186° on admixture with a specimen of allopregnanediol-3(β),20(β)-one-16 diacetate (V) (m.p. 185–186°) prepared from compound IV. Transmission measurements in the infra-red region are given in Fig. 2 (Curve D). Although generally in good agreement with Curve C, they show a shoulder at 5.91 μ. This may be due to the presence of some Δ^{17-20}-unsaturated 16-ketone which could form from V by the elimination of acetic acid. Such an impurity could account, at least in part, for the high carbon value found for the material recovered from the infra-red measurements.

Analysis (E. H.)—(Sample 1.5 mg.) $C_{25}H_{38}O_5$. Calculated. C 71.74, H 9.15
Found. " 72.54, " 9.27

Reduction of Allopregnanediol-16(α),20(β)-one-3 Diacetate (VIII); (a) In Alcohol—8.5 mg. of compound VIII, 13.9 mg. of platinum oxide, and 4.5 cc. of 95 per cent ethanol were shaken with hydrogen for 2 hours. A solution of the reaction product and of 32.2 mg. of digitonin in 3.2 cc. of 80 per cent ethanol yielded only a very small amount of precipitate. The mixture was, therefore, evaporated and freed of digitonin by the pyridine method (54). The ether-soluble material was acetylated with 1 cc. of acetic anhydride in 2 cc. of pyridine at room temperature for 19 hours. The resulting triacetate after five recrystallizations from methanol melted at 170–171° (4.3 mg.). Admixture with compound I did not depress the melting point.

Analysis (E. H.)—$C_{27}H_{42}O_6$. Calculated, C 70.10, H 9.15; found, C 70.26, H. 9.13

(*b*) *In Acid*—14.8 mg. of platinum oxide suspended in 3 cc. of glacial acetic acid were reduced with hydrogen. 24.7 mg. of compound VIII in 4 cc. of this solvent and 0.1 cc. of 48 per cent hydrobromic acid were added and shaking continued for 105 minutes. The reaction product was acetylated as in procedure (*a*). 14.7 mg. of platelets were obtained which melted at 170–171° and at 170.5–171.5° when mixed with allopregnanetriol-3(β),16(α),20(β) triacetate (I). Rotation, $[\alpha]_D^{24} = -47°$ ($c = 0.5$).

3,16-Dichloroallopregnanol-20(β) Acetate (X)—99 mg. of dry calcium carbonate (low in alkali) were added to a solution of 48.3 mg. of allopreg-

nanetriol-3(β),16(α),20(β) 20-monoacetate (II) in 8 cc. of dry chlorofo
which had been freed of alcohol. The suspension was kept in ice wh
140 mg. of phosphorus pentachloride were added in small portions
minutes) and then allowed to stand at 25° for 1 hour. The excess reag
was hydrolyzed by the addition of 8 cc. of a cold 8 per cent sodium
carbonate solution. The mixture was acidified and extracted with eth
The organic phase was washed repeatedly with dilute hydrochloric ac
with sodium carbonate solution, and with water, and taken to dryn
(54.2 mg.). The reaction product, which was readily soluble in cold be
zene, was recrystallized from methanol and from ethanol. 33.7 mg.
colorless heavy plates were obtained which melted at 202–206° with so
decomposition. Further recrystallization from ethanol or from aceto
did not raise or sharpen the melting point.

Analysis (*E. H.*)—$C_{23}H_{36}O_2Cl_2$. Calculated, C 66.49, H 8.74; found, C 66.55, H 8

Chromatographic separation of the mother liquors and recrystallizati
from ethanol yielded an additional 2.2 mg. of the dichloride (total yie
68 per cent).

Reduction of 3,16-Dichloroallopregnanol-20(β) Acetate (X); Allopregnan
20(β) Acetate (XI); (a) With Sodium—230 mg. of sodium were added
small pieces to a solution of 24.5 mg. of compound X in 4 cc. of propan
which was heated under a reflux until all the sodium had reacted (
minutes). The mixture was cooled and taken up in ether and was wash
repeatedly with water. The ether solution on evaporation yielded 18
mg. of a crystalline residue which was reacetylated at room temperatu
(2 cc. of pyridine, 1 cc. of acetic anhydride for 14.5 hours). The aceta
after recrystallization from 95 per cent ethanol melted at 155–158° (18
mg. corresponding to a yield of 88 per cent). Further recrystallizati
raised the melting point to 158–159.5°. It remained unchanged on a
mixture of a specimen of allopregnanol-20(β) acetate (m.p. 158–160
prepared from allopregnanone (XII).

Analysis (*E. H.*)—$C_{23}H_{38}O_2$. Calculated, C 79.71, H 11.05; found, C 79.76, H 11
Rotation, $[\alpha]_D^{26} = +34°$ (c = 0.5, acetone)

The melting point recorded for this compound is 156° (11).

12.0 mg. of allopregnanol-20(β) acetate (XI) (prepared from compou
X), 2.1 cc. of 95 per cent alcohol, and 0.4 cc. of an aqueous solution
sodium hydroxide (4 per cent) were heated under a reflux for 2 hou
The mixture was distributed between ether and water. The residue fro
the washed ether layer was recrystallized repeatedly from dilute ethan
and from petroleum ether. The final product, a reference specimen
allopregnanol-20(β), and a mixture of both all melted at 142–143°, wh

he resolidified melts liquefied at 149°. This change in melting point vas frequently but not invariably observed. Occasionally the higher nelting product was obtained by recrystallization. The phenomenon s ascribed to dimorphism. Allopregnanol-20(β) has been reported to melt at 140° (11).

(b) *With Nickel*—A solution of 6.0 mg. of compound X in 5 cc. of alcohol and 0.5 cc. of a suspension of a freshly prepared W-7 nickel catalyst (9) (about 300 mg.) were shaken with hydrogen for 3 hours. The catalyst was removed and the solution distributed between ether and water. The ether layer was washed with dilute hydrochloric acid and repeatedly with water and evaporated. The residue was recrystallized three times from 95 per cent ethanol. The final product (3.9 mg.) melted at 157–159° and at 158–159° when mixed with a sample of allopregnanol-20(β) acetate prepared from allopregnanone. In a similar experiment (in the presence of added sodium hydroxide) with a W-4 catalyst that had been stored under ethanol for 7 months only a trace of ionizable chlorine was formed and most of the dichloride X was recovered unchanged.

Preparation and Reduction of Allopregnanone-20 (*XII*)—Allopregnanedione-3,20 was converted to allopregnanone-20 by a modification of the procedure of Marker and Lawson (11). Details are given, since we obtained a higher melting product in better yield. 100.4 mg. of allopregnanedione, 2.5 gm. of zinc (30 mesh), and 8.3 cc. of 95 per cent alcohol were heated in an Erlenmeyer flask fitted with a reflux condenser. When all the diketone had dissolved, 3.5 cc. of a 1:1 mixture of alcohol and concentrated hydrochloric acid were added in nine equal portions during 25 minute intervals; heating was continued for another 40 minutes (total 4 hours). The reaction mixture was allowed to cool and its liquid phase was distributed between ether and dilute hydrochloric acid. The organic layer was washed again with acid and repeatedly with water and yielded on evaporation 96.0 mg. of crystalline residue. This was dissolved in 10 cc. of petroleum ether containing 20 per cent of benzene and passed through a column (66 × 8 mm.) of alumina. Elution with 5, 10, 20, and 40 cc. of this solvent mixture yielded 37.0, 42.4, and 9.2 mg. of crystals and 0.9 mg. of an oil (Fractions *a* to *d*, respectively). Continued elution with 2:1 mixtures of benzene and petroleum ether and with pure benzene gave a total of 6.2 mg. of crystalline material. These fractions (*e* to *g*) gave little or no color in the dinitrobenzene reaction when tested at a concentration of 100 γ or more per 0.2 cc. Fraction *a* likewise contained a non-chromogenic impurity which was removed by rechromatographing. Recrystallization from methanol yielded 57.3 mg. of etched platelets melting at 136–138° and 10.6 mg. of less pure material melting at 133–135.5°. If this is included, the yield was 71 per cent. The non-chromogenic nature of the impurities encountered suggests that furt reduction of the reaction time or greater dilution of the acid m be beneficial.

Analysis (*E. H.*)—$C_{21}H_{34}O$. Calculated, C 83.38, H 11.33; found, C 83.25, H 11

Absorption data on the colored solution obtained with allopregnano 20 in the dinitrobenzene reaction are given in Curve 5 of Fig. 3. Wh the crude reaction product was recrystallized from methanol, it ga 81.5 mg. of plates melting at 130–133°. Purification of this material continued recrystallization, however, proved less efficient than chroma graphic separation. Marker and Lawson obtained a preparation of al pregnanone-20 melting at 129° in 55 per cent yield.

Allopregnanone-20 (XII) was reduced with platinum in acetic a (11) at normal pressure. The crystalline reaction product was acet lated at room temperature to yield allopregnanol-20(β) acetate (XI). R tation, $[\alpha]_D^{23} = +38°$ ($c = 0.5$, acetone). The free alcohol was obtain by hydrolysis of the acetate as described above or directly by recryst lization of the reduction product of allopregnanone.

Preparation of Triacetates of Allopregnanetriol-3(β),16(β),20(β) (V and of Allopregnanetriol-3(β),16(β),20(α) (XX) from Tigogenin Acet (XIIIb)—Diosgenin was isolated (56, 57) from beth root (S. B. Peni and Company), acetylated with pyridine and acetic anhydride at roo temperature, and converted to tigogenin acetate either by the procedu of Marker *et al.* (56) or by reduction of an alcoholic solution with a p ladium-calcium carbonate catalyst (58). The conversions of tigogen acetate to the two isomeric allopregnanetriol triacetates were effect essentially by the methods of Marker and his coworkers (19–21). T resulting triacetates were purified by chromatographic separation.

Allopregnanetriol-3(β),16(β),20(β) triacetate (*VI*) melted at 164–16 Rotation, $[\alpha]_D^{25} = +48°$ ($c = 0.7$). Measurements of its infra-red tran mission spectrum are reported in Curve B of Fig. 2. The melting poin reported for this compound are 161–163° and 161.5–163° (20).

Allopregnanetriol-3(β),16(β),20(α) triacetate (*XX*) showed a melti point of 166.5–168°. Rotation, $[\alpha]_D^{32} = +25°$ ($c = 0.8$).[8] The melti point given in the literature is 166° (21).

48 Hour Solvolysis of Allopregnanetriol-3(β),16(β),20(β) Triacetate (V —This experiment was carried out simultaneously with the 48 hour s volysis of compound I and aliquots of the same alkali solution were us in both. 29.9 mg. of compound VI and an equimolar amount of pot sium hydroxide were kept in methanol (total volume, 10.45 cc. to give

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[8] The negative value published previously (59) is a printer's error.

.00618 M solution) at 20° ± 0.1° for 48 hours. The mixture was then listributed between 80 cc. of ether and 42 cc. of water. The ether phase vas washed twice more with water and yielded on evaporation 27.5 mg. f a crystalline residue (average molecular weight found 425; calculated or diacetate, $C_{25}H_{40}O_5$, 420.6). Extraction with benzene yielded 25.3 ng. of soluble material which was recrystallized from dilute and from bsolute methanol and repeatedly from acetone. 5.4 mg. of needle-haped crystals melting at 209–210° were obtained in addition to 8.9 mg. f less pure diacetate melting at 200–206° (yield 53 per cent).

Analysis (*E. H.*)—$C_{25}H_{40}O_5$. Calculated, C 71.39, H 9.59; found, C 71.33, H 9.47

48 Hour Solvolysis of Allopregnanetriol-3(β),16(β),20(α) Triacetate (*XX*)—A solution of 37.6 mg. of the triacetate XX and of an equimolar amount of potassium hydroxide in 13.15 cc. of methanol (resulting molarity 0.00618) was kept at 20° for 48 hours and distributed between ether and water. The neutral reaction product (33.8 mg.; average molecular weight found 416; calculated for diacetate, $C_{25}H_{40}O_5$, 420.6) upon recrystallization from methanol and from acetone yielded 21.0 mg. (61 per cent) of the 16,20-diacetate as elongated platelets melting at 228–232°.

Analysis (*E. H.*)—$C_{25}H_{40}O_5$. Calculated, C 71.39, H 9.59; found, C 71.27, H 9.51

Oxidation of Allopregnanetriol-3(β),16(β),20(β) 16,20-Diacetate (*XVI*)—A solution of 6.3 mg. of compound XVI (m.p. 204–208°) in 1.6 cc. of acetic acid was mixed with a solution of 1.5 mg. of chromium trioxide in 0.13 cc. of 90 per cent acetic acid and kept at 25° for 3 hours. The neutral oxidation product (6.2 mg.), which was isolated in the usual manner, failed to crystallize. An aliquot was studied in the dinitrobenzene reaction which showed the same color changes as those described below (Fig. 3, Curve 4). The remainder of ketone XVII was dissolved in 5 cc. of 80 per cent ethanol containing 50 mg. of sodium hydroxide and heated for 1 hour under a reflux. The hydrolyzed product (4.8 mg.) was crystalline. Examination of its ultraviolet absorption spectrum revealed that it was essentially (about 98 per cent) free of α,β-unsaturated ketones. After purification by means of Girard's reagent and recrystallization from acetone the preparation of allopregnanediol-16(β),20(β)-one-3 (XVIII) melted at 241–246° with decomposition.

Analysis (*E. H.*)—$C_{21}H_{34}O_3$. Calculated, C 75.40, H 10.25; found, C 75.08, H 10.14

Oxidation of Allopregnanetriol-3(β),16(β),20(α) 16,20-Diacetate (*XXI*)—21.0 mg. of compound XXI were oxidized for 4 hours with 5 mg. of chromium trioxide in 1.45 cc. of 98 per cent acetic acid at 25°.

The neutral reaction product (19.7 mg.) was recrystallized repeate from methanol and from petroleum ether. The ketodiacetate X melted at 192–193°.

Analysis (*E. H.*)—$C_{25}H_{38}O_5$. Calculated, C 71.74, H 9.15; found, C 71.80, H

Its behavior with alkaline dinitrobenzene is typical of 3-ketosteroi The solution turned purple immediately and started to become bro after about 15 minutes. The color change was complete after about minutes. The absorption characteristics of the final product are giv in Fig. 3, Curve 3.

Precipitability with Digitonin—The 16,20-diacetates VII, XVI, a XXI were dissolved in 80 per cent ethanol to give 1 per cent solutio allopregnanetriol-3(β),16(α),20(β) to give a 0.25 per cent soluti Equal volumes of a 1 per cent solution of digitonin in 80 per cent etha were added. Compound XXI gave a flocculent precipitate after 15 m utes; the other mixtures remained clear for the first 24 hours. On t 2nd day the two other diacetates gave small amounts of flocculent p cipitates, while the triol from mare urine yielded some crystalline mater having the appearance of the original triol.

Infra-Red Measurements—The instrument used was a Perkin-Elm recording spectrometer with sodium chloride prism and with D.C. ampli cation. Glass and lithium fluoride shutters were employed. The recor ings in the region from 12 to 15 μ have not been plotted, since compou V and particularly compound VI at the concentration studied show very little absorption.

SUMMARY

Evidence has been presented to show that the allopregnanetriol-3,16, from the urine of pregnant mares is to be designated as allopregnanetri 3(β),16(α),20(β), the isomer obtained from tigogenin by means of pe sulfuric acid as allopregnanetriol-3(β),16(β),20(α), and the one prepar from pseudotigogenin as allopregnanetriol-3(β),16(β),20(β). The co figuration of the carbon-oxygen bond at C-16 of the sapogenins and the reaction products has been discussed.

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HE RELATION OF CHEMICAL STRUCTURE TO THE STIMULATION OF LIPIDE PHOSPHORYLATION*

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It has been reported recently that the administration of a single dose f ethanolamine (2, 42) or of its products of partial methylation (2) stimuates the formation of total phospholipides in the liver and the intestine f rats on a low protein diet. This effect is similar to that which had been bserved after feeding choline (38, 3) or compounds like betaine (39) and ethionine (40) which contain transferable methyl groups and therefore ay contribute to the synthesis of choline in the body. We have now nvestigated a number of other compounds in an attempt to elucidate the elationship of the chemical structure of a substance to its action on lipide hosphorylation. The compounds tested included analogues of ethanolmine (di- and triethanolamine), the mono- and diethyl derivatives of thanolamine, the triethyl analogue of choline, primary and tertiary alihatic amines (ethyl-, methyl-, and trimethylamine), and ammonium hloride. In a few additional experiments the action of some other subtances, which may be of biological interest, has been also investigated. These included serine and inositol, which are constituents of certain tissue hospholipides, glutathione, and dimethylaminoazobenzene (butter yellow). t has been postulated that this azo dye acts as a methyl donor (24) and hat its carcinogenic action may have some relation to this property.

EXPERIMENTAL

As in previous experiments, male albino rats (100 to 110 gm.) were divided in groups of three to six and maintained for 7 days on a low fat, low protein diet (Diet 26 (3)) in which a solution of pure B vitamins (17) was incorporated daily. In two experiments on butter yellow, the azo dye was also added to the diet. In part of the experiments on choline and riethylcholine a diet with a higher fat content was employed (Diet 28,

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containing casein 5 parts, dextrin 28, sucrose 29, Crisco 30, cod liver Ruffex 2, salt mixture (37) 4).

As a rule, one or two of the animals in each group received water, the others were given the substances to be tested, each rat receivi different compound. Thus, while the same rat was used as a contro more than one of the substances, for any given substance each test an had its own control in the same group. Only in the experiments on bu yellow separate groups of test and control rats were run simultaneousl

The water (1 cc.) and the compounds tested (0.2 mM in 1 cc. of wa were given in one dose by stomach tube, except in the experiments on action of glutathione, in which the intraperitoneal route was used in l control and test animals. 5 minutes later all rats were injected in peritoneally with a solution of Na_2HPO_4 containing radioactive phospho and after 6 hours they were killed by decapitation. On the lipides tracted from the liver and the small intestine the radioactivity and pl phorus were determined, as previously described (3).

Triethylhydroxyethylammonium chloride (triethylcholine) was ge ously supplied by Dr. C. C. Lucas of the University of Toronto. The ot substances were commercial products. The ethanolamines were purcha as the bases, and their identity and purity (not less than 95 per cent) w checked by determinations of specific gravity, boiling point, acid equi lent, ammonia evolved by alkaline periodate, and formol titration. P to the administration to the animals, the solutions were neutralized to 5.5 with HCl. The amines and choline were obtained as the chlorides, titer of the solutions fed to the animals being estimated on the basis determinations of total N and Cl.

Results

For the sake of brevity, in Tables I to IV we are reporting only the av age values for the radioactivity and the specific activity of the lipide ph phorus. The statistical evaluation of the data has been made by apply the *t* test of significance to the differences between the average values the test animals and those of the corresponding controls. In the exp ments reported in Tables I and IV, the average of the data obtained wit given substance (four to seven rats) was compared with the general aver of the controls in the various groups (liver, twenty-one rats; intesti twenty rats). In addition, in all experiments, except those on dimeth aminoazobenzene, another method of comparing the data was also us The differences observed in the various experiments between the rat ceiving a certain compound and the controls of the same group receiv water were averaged. The value of *t* for each average difference was th calculated according to the formula for testing the significance of a sin an. While some discrepancies are apparent, in most cases the results culated by either method were in fairly good agreement with each ıer.

TABLE I

Action of Various Compounds on Lipide Phosphorylation in Liver of Rats

The rats were maintained for 7 days on a low protein, low fat diet (Diet 26).

. of ts	Substance given	Liver lipides, averages			
		Radioactivity		Specific activity	
		Per cent of dose injected × 100	Per cent change	× 100	Per cent change
21	Water (controls)	414 ± 17		90 ± 3	
7	Diethanolamine	701 ± 34	+69* (+65*)	144 ± 13	+60* (+58*)
5	Triethanolamine	536 ± 43	+30* (+35*)	108 ± 8	+20† (+29*)
5	Ethylethanolamine	759 ± 40	+83* (+87*)	150 ± 6	+66* (+65*)
5	Diethylethanolamine	781 ± 41	+89* (+93*)	148 ± 12	+65* (+63*)
7	Ethylamine	480 ± 41	+16 (+20†)	114 ± 7	+27* (+23†)
5	Methylamine	428 ± 32	+4 (+10)	88 ± 8	−2 (+9)
5	Trimethylamine	470 ± 47	+14 (+21)	91 ± 7	+1 (+12)
5	Ammonium chloride	407 ± 29	−2 (−2)	87 ± 5	−3 (0)
4	DL-Serine	379 ± 29	−8 (−15)	86 ± 6	−5 (−8)
5	*i*-Inositol	447 ± 8	+8 (0)	90 ± 8	0 (−5)
4	Glutathione	419 ± 55	+1 (+24)	94 ± 4	+4 (+11)

The figures preceded by ± are the standard error of the mean. "Radioactivity" presents the per cent of the dose injected which was found in the lipides of the hole liver of the experimental animals. "Specific activity" represents the radio-tivity divided by the mg. of lipide P calculated for the whole liver of a 100 gm. t. The figures have been multiplied by 100 in order to make the values directly mparable with those reported in previous papers from this laboratory, in which e radioactivity was expressed in "relative radioactive units" (r.r.u.), the dose jected being assumed to be = 10^4 r.r.u. The figures in parentheses indicate the er cent change calculated on the basis of the average difference between the test nimal and the control of the same experimental group. Unmarked values have a robability of chance occurrence $P > 0.05$.

* $P < 0.01$.

† $P < 0.05$.

From Table I it is apparent that diethanolamine, triethanolamine, ethyl-thanolamine, and diethylethanolamine caused quite significant increases both the total radioactivity and specific activity of the liver lipides. he per cent increases are of the same order of magnitude as those which ave been found previously after giving ethanolamine, methylethanolamine, or dimethylethanolamine (2). Among the other substances l in Table I, only ethylamine caused increases which were statisticall nificant. In some of the experiments in which methylamine, trime amine, or glutathione was tested, appreciable increases over the con were found. But, because of the variability in the individual values a the small number of experiments, the average differences are not sig cant, and the only conclusion which can be drawn at present is that t substances are either ineffective or very slightly effective.

The same statement should be made for the results obtained with bu yellow (Table II). Since this substance is insoluble in water, either it

TABLE II

Action of Dimethylaminoazobenzene on Lipide Phosphorylation in Liver of R

The rats were maintained for 7 days on a low protein, low fat diet (Diet 26).

No. of rats	Substance given	Liver lipides, averages			
		Radioactivity		Specific activity	
		Per cent of dose injected × 100	Per cent change	× 100	Per cha
8	Water (controls)	441 ± 30		90 ± 6	
7	Dimethylaminoazobenzene*	576 ± 36	+32†	96 ± 5	
5	" ‡	518 ± 17	+17	101 ± 2	+

See the explanations below Table I.

* 60 mg. of the substance suspended in 1 cc. of water were given by stomach 5 minutes before injecting the isotopic phosphate.

† $P < 0.05$.

‡ Dimethylaminoazobenzene was added to the diet (100 mg. per 100 gm. of d In addition, 60 mg. of the substances suspended in 1 cc. of water were given by s ach tube 5 minutes before injecting the isotopic phosphate.

given as a water suspension in one dose (eight rats) or it was incorpor in the daily ration of the animals for a number of days and an additi dose, suspended in water, was administered immediately before the in tion of P^{32} (five rats). It is apparent that in neither type of experin were conditions exactly comparable with those under which all other c pounds were tested, and that a slow absorption of the azo dye might account for our failure to obtain more marked and consistent increase the phosphorylation of liver lipides.

Table III summarizes the results of experiments in which the actio the triethyl analogue of choline and that of choline itself on the phospho ation of the liver lipides have been compared. Since previous results shown that the action of choline on lipide phosphorylation is enhance e simultaneous administration of a large amount of fat, in some of these periments, instead of Diet 26, a diet with a higher fat content was em- ›yed. In the liver of the controls on the high fat diets, the specific tivity of the lipide P was appreciably lower than in the corresponding imals on Diet 26. After administration of the test substances, the indi- dual data exhibited a considerable spreading and overlapping with the ntrols. However, on both diets, the average values for the test animals e considerably higher than those found in the respective controls, some- ıat larger increases being obtained in the rats which were maintained on e high fat diet and which received the triethyl analogue.

TABLE III

Comparison between Action of Triethylcholine and Choline on Lipide Phosphorylation in Liver of Rats on Low and High Fat Diets

The rats were maintained for 7 days on the experimental diets.

Diet No.	No. of rats	Substance given	Liver lipides, averages			
			Radioactivity		Specific activity	
			Per cent of dose injected × 100	Per cent change	× 100	Per cent change
(Low fat)	8	Water (controls)	456 ± 28		91 ± 5	
	7	Triethylcholine	639 ± 51	+40* (+25*)	106 ± 3	+16† (+17†)
	8	Choline	493 ± 23	+8 (+11)	105 ± 3	+15† (+14†)
(High fat)	6	Water (controls)	428 ± 27		70 ± 3	
	6	Triethylcholine	607 ± 49	+42* (+42*)	90 ± 11	+30 (+31)
	6	Choline	568 ± 22	+33† (+46†)	85 ± 3	+22† (+31*)

See the explanations below Table I.

* $P < 0.05$.

† $P < 0.01$.

In Table IV are reported the results obtained on the lipides of the small ıtestine. The fact that somewhat higher values were observed after dministration of those compounds which appeared to be most effective ›r the stimulation of lipide phosphorylation in the liver may be suggestive. ‹s a rule, however, in the intestine the differences between test and control nimals were not statistically significant. In this respect we should like to oint out again ((3) foot-note 5) that our determinations were carried out n the whole intestine and therefore included the lipides of the muscle as ell as those of the mucosa. In the former tissue the rate of lipide phos- horylation is slower, and possibly it is affected by the substances admin- ıtered less markedly than in the mucosa. One might, therefore, postulate that more extensive changes would have been detected if the values been estimated on the lipides of the isolated mucosa.

Table IV

Action of Various Compounds on Lipide Phosphorylation in Small Intestine Rats

The rats were maintained for 7 days on a low protein, low fat diet (Diet 26).

No. of rats	Substances given	Intestinal lipides, averages			
		Radioactivity		Specific activity	
		Per cent of dose injected × 100	Per cent change	× 100	Per cent change
20	Water (controls)	164 ± 9		54 ± 2	
7	Diethanolamine	168 ± 18	+2 (−3)	61 ± 3	+13 (
5	Triethanolamine	155 ± 14	−5 (+20)	59 ± 5	+9 (+
5	Ethylethanolamine	199 ± 20	+21 (+36)	63 ± 3	+17* (+
5	Diethylethanolamine	162 ± 28	−1 (+11)	58 ± 4	+7 (
7	Ethylamine	164 ± 17	0 (+14)	63 ± 3	+17 (+
5	Methylamine	178 ± 16	+9 (+5)	57 ± 5	+6 (+
5	Trimethylamine	159 ± 12	−3 (−6)	55 ± 3	+2 (+
5	Ammonium chloride	161 ± 13	−2 (−13)	55 ± 3	+2 (−
2	DL-Serine	129 ± 2	−21 (−30)	48 ± 3	−11 (−
2	*i*-Inositol	133 ± 9	−7 (−19)	54 ± 5	0 (
4	Glutathione	111 ± 10	−33 (−23)	44 ± 2	−19 (−

See the explanations below Table I.
* $P < 0.05$.

DISCUSSION

In the present study, as in previous similar investigations on the lip phosphorylation, the amount and concentration of P^{32} in the lipides control animals are compared with the values found in animals to whic substance has been given. A significant increase in these values is tal as a *qualitative* indication for a stimulation of the lipide phosphorylati

The question may be raised whether more definite or more reliable c clusions could be drawn if the radioactivity values were expressed as "r tive specific activities;" that is, as the ratios of the specific activity of lipide P to the specific activity of the inorganic or acid-soluble phosphor

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[1] These ratios do not express adequately the relationship between the isot concentrations in a compound and in its precursor. The specific activity of a c pound at any given time is dependent upon the values of the specific activity of precursor during the *whole* time interval between the introduction of the isotope the time at which the determinations are carried out. In other words, in a cor calculation, the *area* of the specific activity-time curve of the precursor, rather t the *level* reached at the end of the experiment, should be taken into account (

some of our present experiments the specific activity of the inorganic iosphate has also been determined. Since, within the limits of error of .r methods, identical values were obtained in the control and test animals, ese findings may be considered in line with the reasonable assumption at the substance administered did not modify substantially the dis-ibution and the exchanges of the isotope in the inorganic P fraction. In her experiments, carried out in this laboratory as part of a separate udy, simultaneous determinations on the inorganic and lipide fractions ere also made on the livers of rats killed at various intervals after the gestion of P^{32}. In these determinations, the average specific activity of e lipide P exhibited a standard deviation of 13 per cent at the 3rd hour id 14 per cent at the 6th hour, while the corresponding standard devi-ions of the average relative specific activity were 15 and 16 per cent. The ialysis of some of the data reported by Zilversmit *et al.* in their study on ie formation of phospholipides in the intestine (59) is even more informa-ve. 15 minutes after the introduction of P^{32} in both fat-fed and control its, the specific activity of the phospholipide P showed a very great vari-bility, while the individual variations were much less extensive when the alues were expressed as relative specific activities. In the 1 hour experi-ents, the standard deviations of the mean specific activity of the lipide P ere 36 per cent (fat-fed animals) and 27 per cent (controls), as compared ith standard deviations of 24 and 19 per cent for the relative specific ctivities. However, in the rats killed 6 hours after the P^{32} was admin-tered, the standard deviations of the averages were 11 per cent (fat-fed) id 6 per cent (controls) for the specific activity of the lipide P, while the orresponding values for the relative specific activity were 16 and 8 per ent, respectively. It seems that the use of relative specific activities is efinitely advantageous in experiments made at the earlier times after the troduction of isotope, when the specific activity of the inorganic P is

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his point seems especially important when considerable and rapid changes in the pecific activity of the precursor take place, as after the administration of P^{32}. It iould be noted also that neither the site of the synthesis of phospholipides nor the entity of their precursors is known. On the assumption that the immediate pre-irsor is formed very rapidly from the inorganic phosphate in the cell, the specific ctivity of the intracellular phosphate would represent a reliable basis for the cal-ilation of the "relative specific activity." However, the inorganic phosphate, as etermined usually in a tissue, includes both extra- and intracellular phosphate. ecause of the difficulty in determining separately the latter fraction, it has been iggested that the total acid-soluble P be used (59). This suggestion is question-ble, since the acid-soluble P represents a quite heterogeneous mixture of a large umber of compounds, presumably formed at various rates. The hypothetical hospholipide precursor is probably included in this fraction, but, since it may epresent only a minor component, its specific activity could be very different from iat of the whole mixture (4).

decreasing rapidly and the rôle of other factors (such as variations ir rates of absorption of the isotope from the site of injection and of its p tration into the cells) may be considerable. In the longer intervals importance of these factors probably becomes less marked and one m expect that the extent of the individual variations in the specific acti of the lipide P would not be appreciably lowered by expressing the valu relative specific activities.

If the above considerations are correct, the present results, in conj tion with those previously obtained in this laboratory (2), show ethanolamine, the two analogues and the four alkyl derivatives of etha amine which we have tested, and, in addition, choline and its trie analogue, stimulate the lipide phosphorylation in the liver (some poss also in the intestine). These compounds are all active, irrespective of nitrogen present as a primary, secondary, or tertiary amine or as quatern ammonium. Furthermore, the activity remains the same if ethyl gro are substituted for the methyl groups linked to the nitrogen. It app that the presence of an alcoholic hydroxy group is important, since NI and the various simple amines tested, with the exception of ethylam showed less activity or no activity.

It is also of interest to compare the action of various substances lipide phosphorylation with other biological actions exerted by the s compounds. Such a comparison has been made in Table V, which am fies a similar table presented by Moyer and du Vigneaud (36). It wil noted that the stimulation of the formation of phospholipides in the l has a much lower degree of specificity than the other actions studied cluding the lipotropic action. This further confirms a statement previo made on the basis of the results obtained after administration of etha amine and of its products of partial methylation (2). It was then poi out that, while all substances with a definite lipotropic action have l shown to cause an increase in lipide phosphorylation, the latter effe exhibited also by compounds which are not clearly lipotropic.

At present a discussion on the mechanisms through which the var substances enhance the formation of phospholipides in the liver can onl highly speculative. It is likely that choline and ethanolamine are ac because they are primarily used as intact molecules for the synthesi the two main types of phospholipides. Other compounds may be effec by contributing to the synthesis of choline, either as methyl donors, as methionine or betaine, or as methyl acceptors. In the last categ ethanolamine will also be included and, in addition, methyl- and dimet ethanolamine. The finding of little or no stimulation by methylan trimethylamine, and sarcosine would be in line with the concept that methyl groups of these compounds are not easily transferable in the or

n. Serine and inositol are also constituents of certain phospholipide ictions. Since, however, these fractions represent only a small proportion the total phospholipides of the liver, an increased formation of these ictions after administration of serine or inositol would not be easily tected when the determinations are carried out on the total phospholipide ixtures. As for the other substances which we found to be effective and nich are not components of natural phospholipides, the possibility that hylamine might be partly converted into ethanolamine should perhaps considered.[2] Other substances can probably be used directly or inrectly for the formation of phospholipides, analogues of those normally esent in tissues. Indeed, in the tissues of animals fed analogues of ioline, such as arsenocholine (56) or triethylcholine (35), the presence of ese unnatural components has been demonstrated. We have some inrect evidence that diethanolamine can likewise be incorporated into a iospholipide molecule.[3] On the other hand, it is obvious that a more direct mechanism (such as a general increase of the metabolism (20)) iould be postulated for the stimulation of lipide phosphorylation exerted y cystine and cysteine.

Finally, it should be pointed out that in the present and previous experi-

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[2] Such a process would require a partial oxidation of the unsubstituted alkyl oup without detachment of the amino group from the other carbon. Numerous amples of the biological oxidation of methyl groups have been reported. Besides e evidence for the oxidation of a methyl attached to an S atom (as in methionine 3)), or to an N atom (as in sarcosine (23) and in other methyl amino acids (32)), to an aromatic C (as in toluene and in various methyl-substituted benzenes and ienols (58)), the well known observations on the ω oxidation of fatty acids (50) and straight chain or cyclic terpenes and terpene derivatives (31) can be mentioned. the examples last mentioned it is probable that the oxidation of the methyl to e carboxyl group occurs through the intermediate formation of the primary alohol. As was pointed out by Kühn *et al.* (31), while in the oxidation of fatty acids id straight chain terpenes this stage cannot be detected, in the metabolism of clic terpenes the alcohol conjugates with glucuronic acid and thus can be isolated. is apparent that the oxidation of the alkyl group (or at least the possibility of etecting this process) is dependent upon the greater resistance to oxidation of her groups in the molecule. This is perhaps best illustrated by the formation of uconic acid from sorbic acid. While only minute amounts of muconic acid are creted when the free sorbic acid is fed to rabbits, the yield of the corresponding uconic acid derivatives is 300 to 400 times greater after feeding the amide, or the ethylamide, or the anilide of sorbic acid (31). If and to what extent these exames and these views apply to the specific case of the oxidation of ethylamine is, of ourse, a matter of speculation. However, it may be pointed out in this respect iat, while higher aliphatic amines and aromatic alkylamines are actively oxidized id deaminated by tissue slices and extracts, under the same conditions ethylamine s well as propyl- or methylamine) causes very little or no increase in the oxygen onsumption and in the production of ammonia (43, 9).

[3] Artom, C., Cornatzer, W. E., and Crowder, M., unpublished experiments.

Table V

Comparison between Stimulation of Lipide Phosphorylation and Other Biological Actions of Various Compounds

The figures in parentheses refer to the bibliography.

Compound	Rats or mice						Chickens	
	Lipide phosphorylation, liver	Lipotropic action, liver	Antihemorrhagic action, kidney	Synthesis or replacement of ethanolamine*	Synthesis or replacement of choline*	Synthesis of methionine†	Growth	Antiperotic action
DL-Alanine	− (40)	− (6)						
L-Aspartic acid	− (40)‡	− (6, 14)						
Betaine	+ (39)	+ (8)	+ (21)	− (46)	+ (46, 55)§	+ (52, 11)	± (1, 30)	− (30)
Choline	+ (38, 3)	+ (7, 8)	+ (22)	− (46)	+(12)	+ (52, 44)	+ (25)	+ (25)
Creatine	± (40)	− (41)	± (21)			− (51)	± (26)	− (26)
L-Cysteine	+ (38)	− (45)	− (19)					
L-Cystine	+ (38)	− (5)	− (22)					
Diethylethanolamine	+ (‖)	− (10)	± (10)					
Dimethylaminoazobenzene	± (‖)		+ (24)					
Dimethylethanolamine	+ (2)	+ (53)	+ (53)		+ (53)	− (53, 11)	+ (28)	+ (28)
Ethanolamine	+ (2, 42)	− (8)		+ (46, 47)	+ (46, 47)	− (52)	− (26)	− (26)
Glutathione	− (‖)	− (21)	− (21)					
L-Glutamic acid	− (40)	− (5, 14)						
Glycine	− (40)	− (5)		+ (46)				
i-Inositol	− (‖)	+ (18)						
DL-Methionine	+ (38)	+ (49)	+ (22)		+ (54)		− (26)	− (26)
Methylethanolamine	+ (2)				+ (53)	− (11)	± (27, 29)	+ (29)
L-Proline	− (40)	− (6, 14)						
Sarcosine	− (40)	− (51)	− (21)		+ (55)¶	− (51)		
DL-Serine	− (40, ‖)	− (5, 14)		+ (48)				

Taurine	− (40)	− (13)	− (21)					
Triethylcholine	+ (‖)	+ (15)	+ (57)		+ (35)**	− (52)	− (25, 30)	− (25, 30)
Trimethylamine	− (‖)	− (34)				− (52)		

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* Isotope techniques (except for triethylcholine).

† As shown directly (experiments *in vitro* with tissue slices and homogenates, or *in vivo* with tracer techniques), or indirectly (growth on a methionine-free diet supplemented with homocystine).

‡ Administered as asparagine.

‖ Evidence presented in this paper.

§ The methyl but not the N of betaine was efficiently utilized for the synthesis of choline.

¶ Methyl of sarcosine transferred to choline, but at a very slow rate.

** Triethylcholine detected by microbiological and chemical techniques.

ments on the stimulation of lipide phosphorylation the results have b generally obtained after administration of a single large dose of the s stances to animals maintained on experimental diets which were defic in choline, possibly also in ethanolamine precursors. An extension of conclusions drawn from the present results to other experimental condit (*e.g.*, the use of more adequate diets or feeding the compounds over a p longed period) would be quite arbitrary.

SUMMARY

A number of compounds were tested for their ability to stimulate lip phosphorylation in the liver and intestine of rats on low protein di Significant increases in the rate of formation of liver phospholipides w obtained after administration of a single large dose of the followi diethanolamine, triethanolamine, ethylethanolamine, diethylethanolami triethylcholine, and ethylamine. These results are compared with th obtained previously after administration, under similar conditions, choline, ethanolamine, methylethanolamine, and dimethylethanolami It appears that the stimulating action of lipide phosphorylation in the li has a lower degree of structural specificity than other biological acti exerted by the same substances.

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THE VITAMIN B_6 GROUP

XVI. D-ALANINE AND THE VITAMIN B_6 CONTENT OF MICROORGANISMS*

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D-Alanine serves as the nutritional equivalent of vitamin B_6 for *Streptoccus faecalis* R and *Lactobacillus casei* in appropriate media (2, 3), whereas alanine is either ineffective or only slightly so under the same condions. The mechanism by which this "unnatural" amino acid exerts its owth-promoting effects is not known. The suggestion (4) that it serves a precursor from which these organisms synthesize vitamin B_6 is not pported by the findings that *Streptococcus faecalis* fails to form detectle amounts of codecarboxylase (pyridoxal phosphate) or cotransninase (pyridoxal phosphate) when grown with DL-alanine in place of itamin B_6 (5, 6).

As one approach to elucidation of the action of D-alanine, direct assays ere made for the vitamin B_6 content of a variety of microorganisms grown the presence and absence of D-alanine or of vitamin B_6. The results f these studies are presented below.

EXPERIMENTAL

Preparation of Dried Cells—The various organisms, cultural conditions, nd references to media used are listed in Table I. All media were preared initially free of vitamin B_6. Where possible, each organism was hen cultured in four modifications of the indicated medium: (*a*) the asal medium, free of vitamin B_6;[1] (*b*) this medium supplemented with DL-alanine (200 mg. per liter); (*c*) this medium supplemented with a level of pyridoxal (0.001 mg. per liter); and (*d*) this medium supplemen with excess pyridoxal (1 mg. per liter). Pyridoxal was added aseptic following sterilization of the medium. For bacteria, 2 liters of each

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[1] Occasionally, *Streptococcus faecalis* or *Lactobacillus casei*, which require either itamin B_6 or D-alanine for growth, would show very slight growth in this medium, dicating presence of contaminating traces of vitamin B_6. In such cases, these races of the vitamin were removed by a pretreatment procedure exactly similar to hat previously employed to remove essential trace elements from bacterial media 8), before supplementation of the medium with DL-alanine. Thus cells of these wo organisms grown in the presence of D-alanine were obtained in a medium which ontained insufficient vitamin B_6 to permit any visible growth in the absence of this -amino acid. Indicated lots of *Lactobacillus arabinosus* also were grown in a medium rigorously freed of vitamin B_6 by pretreatment with *Streptococcus faecalis*.

TABLE I

*Conditions for Culture of Various Organisms and Yields of Dry Cells from Vario Supplemented Basal Media**

Organism	Experiment No.	Bibliographic reference to medium used	Incubation		Supplements per liter of vit B_6-free medium		
					DL-Alanine		Pyridoxal, 0.001 mg.
					None	200 mg.	
					Dry cell yields, gm. per li of medium		
			°C.	*hrs.*			
Lactobacillus arabinosus	1	(7)	30	20	0.54	0.54	0.59
	2	(7)	37	22	0.59†	0.77†	1.5
" *casei*	1	(8)	37	20	0	0.63†	0.32
	2	(8)	37	20	0	1.54†	0.36
Streptococcus faecalis	1	(9)	37	16	0	0.41†	0.84‡
	2	(9)	37	16	0	0.43†	0.90
	3	(9)	37	16	0	0.62†	0.99‡
Escherichia coli	1	(10)	37	48	0.21	0.35	0.27‡
Saccharomyces carlsbergensis	1	(11)	30	120	0	0	3.4
	2	(11)	30	120	0	0	6.1
Aspergillus niger	1	(12)§	30	240	3.5	6.9	4.8
	2	(12)§	30	120	2.8	4.6	4.3
Penicillium notatum	1	(12)§	25	64	4.2	4.3	3.8

* Inocula were prepared by growing the bacteria in 10 ml. of the indicated min B_6-free medium, supplemented with DL-alanine. Cells were then centrifu washed twice in saline, resuspended in 10 ml. of saline. 2 ml. of this suspension used to inoculate each liter of medium. Yeast and molds were grown on 10 ml. agar slants; cells or spores were washed from these with sterile saline, centrifu and washed as above, resuspended, and 2 ml. of the resulting suspension use inoculate each liter of medium.

† Cells were grown in pretreated medium.

‡ Pyridoxamine in place of pyridoxal at the same level.

§ $TlCl_3$ omitted.

ture medium yielded sufficient cells for analysis. 300 to 500 ml. of ium sufficed with yeasts and molds. Yeast and mold cultures shaken during incubation; the bacterial cultures were not.

Bacterial and yeast cells were harvested by running cell suspens through a Sharples centrifuge either immediately following incuba or after storage overnight at 5°. The cells were washed by twice susp in 40 ml. of cool saline, shaking vigorously, and centrifuging. The ne from the second washing was removed, and the cells frozen by im- rsing in an acetone-dry ice mixture. They were then dried *in vacuo* m the frozen state. Mold mycelia were filtered from the medium, shed, and similarly dried.

TABLE II

Vitamin B_6 Content of Dried Cell Preparations

Organism	Experiment No.*	Additions per liter of vitamin B_6-free medium			
		None	DL-Alanine, 200 mg.	Pyridoxal, 0.001 mg.	Pyridoxal, 1.0 mg.
		Vitamin B_6 content, γ per gm. of dry cells†			
ctobacillus arabinosus	1	0.98	0.98	1.6	22
	2	0.11‡	0.06	0.10	52
" *casei*	1		0.062‡	2.3	21
	2		0.040‡	1.3	21
eptococcus faecalis	1		0.023‡	0.72§	
	2		0.018‡	0.23	
	3		0.030‡	0.44§	
cherichia coli	1	42	33	43§	
ccharomyces carlsbergensis	1			1.6	15
	2			2.2	14
pergillus niger	1	9.6	12	6.1	
	2	13	24	11	
nicillium notatum	1	3.9	3.8	4.5	

* See Table I.

† Expressed as pyridoxal hydrochloride. Failure to report analyses in the 3rd d 4th columns means that the organisms did not grow in those media (*cf.* Table I).

‡ Cells grown in pretreated medium.

§ Grown with pyridoxamine in place of pyridoxal at same level.

Assay of Cells—Vitamin B_6 was determined with *Saccharomyces carlsber- nsis* 4228 by a slight modification (13, 14) of the method of Atkin *et al.* 1). This organism responds to all three of the known forms of vitamin $_6$. Extracts for analysis were prepared as described by Rabinowitz and ell (13). A few samples, however, contained unusually small amounts vitamin B_6, necessitating the use of a higher ratio of cells to extracting quid than originally recommended. In such cases, the volume of 0.055 HCl was lowered from 180 to 45 ml. per 100 to 250 mg. of sample.

Results

Values found for vitamin B_6 are given in Table II. The amount of the itamin found in cells of lactic acid bacteria varied from very low levels <0.1 γ per gm.) in cells grown in vitamin B_6-free media to very high amounts (up to 50 γ per gm.) in cells from media generously sup mented with the vitamin. This was true both of organisms such *Lactobacillus arabinosus*, which grow in the absence of both D-alanine vitamin B_6, and of organisms such as *Lactobacillus casei* or *Streptoco faecalis*, which grow only when vitamin B_6 or D-alanine is supplied. contrast to the lactic acid bacteria, cells of *Escherichia coli* and *Asper lus niger* are high in vitamin B_6 even when grown in a medium free of vitamin.

Cells of *Streptococcus faecalis* and *Lactobacillus casei* grown w D-alanine in place of vitamin B_6 appear to contain very minute amou of vitamin B_6 (0.06 γ per gm. or less). The accuracy of figures of magnitude is in doubt for the following reasons: (*a*) quantitative ext tion of the vitamin was not assured, since to obtain amounts of the v min sufficient for detection in the assay it was necessary to extract la amounts of cells with a comparatively small volume of acid (*e.g.*, 2000 of cells with 45 ml. of acid); (*b*) to obtain amounts of the vitamin suffic for assay, very large amounts of the extract had to be added to the as tubes, and under such conditions the growth stimulation observed n not have been due solely or even principally to vitamin B_6 present in extracts; (*c*) to avoid non-specific stimulation of the type mentioned ab the assay values were calculated for the most part from a narrow s ment of the standard curve near the origin. Despite this precauti some upward drifts in the assay values at increasing levels of sample w evident, a phenomenon indicative of non-specificity in the assay.

Thus the actual level of vitamin B_6 present in these lots of cells is doubt. Factor (*a*) would favor underestimation of this quantity, fac (*c*) overestimation. It is certain, however, that the amount present is tremely small, a finding which lends no support to the idea that D-alan is serving as a natural precursor for synthesis of the vitamin by these ganisms. Furthermore, it is not certain whether these small amounts vitamin B_6 arise by cellular synthesis or whether they are obtained fr the "vitamin B_6-free" medium. This point is illustrated by compar the amount of vitamin B_6 necessary to produce barely visible growth in basal media used to the total amount of vitamin B_6 found in the c derived from these media in the presence of DL-alanine (Table III). some cases, these amounts of vitamin B_6 could have been present in 1 l of medium without permitting visible growth to take place. Absence visible growth in the unsupplemented medium, however, was the criter used to indicate that the medium was free of the vitamin. Thus methods used, in addition to being of inadequate accuracy at this ra of concentration, are not sensitive enough to differentiate between s thesized and absorbed vitamin B_6. With *Lactobacillus casei*, howe

s unlikely that the amount of vitamin B_6 found in the cells could have
n present in the pretreated medium without being detected. In this
e, limited synthesis of the vitamin is indicated, but the questionable
uracy of the assay values obtained at these low concentrations, dis-
ssed above, prevents a definite decision on this point.

TABLE III

Vitamin B_6 Content of Two Organisms Grown on Media Supplemented with DL-*Alanine*

Organism	Experiment No.	Vitamin B_6 content of cells	Yield of cells	Total vitamin B_6 in cells from 1 liter	Amount of pyridoxal necessary to give barely visible growth
		γ per gm.	*gm. per l.*	*γ*	*γ per l.*
eptococcus faecalis	1	0.023	0.41	0.009	
	2	0.018	0.43	0.008	0.10
	3	0.030	0.62	0.019	
ctobacillus casei	1	0.062	0.63	0.039	<0.05
	2	0.040	1.54	0.062	

TABLE IV

mparative Vitamin B_6 Content of Cells of Saccharomyces carlsbergensis Grown in Presence of Pyridoxal, Pyridoxamine, or Pyridoxine

Additions per liter of medium	Yield of dry cells		Vitamin B_6 content of cells*	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
	gm. per l.	*gm. per l.*	*γ per gm.*	*γ per gm.*
004 mg. pyridoxal	3.4	6.1	1.6	2.5
mg. pyridoxal	14	18	15	14
" pyridoxamine	14	19	7.5	11
" pyridoxine	13	19	23	16

* Expressed as pyridoxal hydrochloride.

With most organisms tested which synthesize large amounts of vitamin
$_6$ there was no evidence of enhanced synthesis in the presence of DL-alan-
ie. *Aspergillus niger*, however, did contain increased amounts of vita-
iin B_6 in its cells when grown in the presence of this amino acid (Table
I).

In two experiments (Table IV), the vitamin B_6 content of yeast grown
the presence of an excess of the three different forms of vitamin B_6 was
ompared. The amount of vitamin B_6 in this organism was consistently
reatest when the organism was grown with pyridoxine, and least with
yridoxamine. When growth of this organism was initiated by the addi-

tion to the medium of a small amount of vitamin B_6 (0.004 mg. per li
it was observed that the vitamin B_6 present in cells from 1 liter of med
surpassed the amount added by about 4-fold. This observation indic
that, on prolonged incubation in the presence of suboptimal quantitie
vitamin B_6, growing cells of *Saccharomyces carlsbergensis* synthe
limited amounts of this vitamin. This observation is similar to thos
Lindegren and Raut (15), who showed that yeast cells grown with lim
amounts of pantothenate synthesize more of this vitamin, but do not g
in its absence. Leonian and Lilly (16) also have pointed out the
with which yeast can be induced to synthesize its own supply of vitan
when incubated for prolonged time intervals.

DISCUSSION

Quantitative results obtained above for the vitamin B_6 content of la
acid bacteria correlate well with the qualitative results of previous stud
Thus Bohonos, Hutchings, and Peterson (17) have shown that *Lactoba*
lus arabinosus synthesizes vitamin B_6 when grown in the absence of an
ternal supply of this vitamin. In this case, vitamin B_6 appeared in
growth medium as well as in the cells. In one case (*Lactobacillus cas*
evidence was cited which indicated that, when this organism was gro
in the presence of an excess of vitamin B_6, it stored the vitamin in exc
of the amounts which it required for growth. Various enzymatic stu
are also in good agreement with our data. Thus Bellamy and Gunsa
found that to produce cells of *Streptococcus faecalis* with high tyros
decarboxylase activity (an enzyme requiring pyridoxal phosphate for
tivity) it was necessary to grow them in a medium which contained mu
larger amounts of vitamin B_6 than were required for maximum grow
and suggested that this was a case of "luxury consumption" of the vi
min to produce cells with optimal functional capacity (18). From
data, it is seen that cells of this organism are much higher in vitamin
when grown with high levels of this vitamin (1 mg. per liter) than wh
grown with much lower levels (0.001 mg. per liter), which are s
sufficient to permit good growth. Similarly, Cohen and Lichstein (
found that cells of this organism grown in the presence of only sm
amounts of vitamin B_6 were unable to decarboxylate tyrosine, but
tained unimpaired the capacity to carry out the transamination reacti
However, when the organism was grown in the complete absence of vi
min B_6 with DL-alanine present to permit growth, they were also una
to catalyze the transamination reaction (6), and this capacity could be
stored to the cells by addition of pyridoxal. Our data show that cells
this organism grown with limiting amounts of vitamin B_6 (0.001 mg.
liter) contain readily measurable amounts of the vitamin; when gro

ith DL-alanine, however, the amounts of the vitamin present are disap-earingly small.

It may be asked whether cells of *Streptococcus faecalis* and *Lactobacillus* *asei* grown with D-alanine require *any* vitamin B_6 for growth and meta-olic activity. This question cannot be decided from the present data. ssay of such cells indicates the presence of traces of vitamin B_6, but limi-ations of the assay method are such that both the accuracy of the figures nd the origin of the small amounts of vitamin B_6 indicated (*i.e.* synthesis y the cells, or absorption from the medium?) are unknown. The pos-ible significance to the organism of these quantities of the vitamin can-ot, however, be discounted on the basis of their small magnitude alone. t is known that the magnitude of the requirement for this vitamin is ighly variable, depending upon the composition of the medium (2, 4, 20, 1), and the small amounts indicated as present in these cells might well e sufficient to fill the greatly reduced metabolic needs, if any, of the or-anism when it is grown in the presence of D-alanine.

It is clear, however, that D-alanine, in permitting growth of *Streptococcus* *aecalis* and of *Lactobacillus casei* in the absence of vitamin B_6, does not func-ion primarily as a precursor from which vitamin B_6 can be synthesized.[2] Rather, presence of this "unnatural" amino acid greatly reduces (and possibly eliminates) the metabolic requirement for the vitamin in the media sed. An alternative explanation for its action would be to consider it s an essential amino acid for these cells, for synthesis of which vitamin B_6 is essential. When D-alanine is supplied preformed, the requirement or the vitamin would then be greatly reduced.

Finally, it should be emphasized that vitamin B_6 and D-alanine are mutually replaceable nutrients for *Streptococcus faecalis* and *Lactobacillus* *casei* only in media of suitably complex nature, such as those used in this investigation. Lyman *et al.* (21) and Speck and Pitt (20) have shown that increased amounts of vitamin B_6 are required for growth of these organisms in the absence of a variety of amino acids which are dispensable when the vitamin is present in excess; *i.e.*, the vitamin is essential for synthesis by the organisms of a number of L-amino acids. If any such amino acids are omitted from the basal media used, then D-alanine will not permit growth in the absence of vitamin B_6.[3] This constitutes additional evidence that D-alanine does not permit synthesis of vitamin B_6, but is consistent rather with the hypothesis that in otherwise complete mec D-alanine replaces vitamin B_6 by supplying an essential product for sy thesis of which vitamin B_6 is normally required. Evidence that this the true explanation for the action of D-alanine will be presented in t following paper of this series (23).

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[2] The possibility that alanine may serve as a precursor for synthesis of vitamin B_6 is not entirely eliminated by these experiments, since the small amounts of vitamin B_6 indicated by assay as present in these cells may have arisen by synthesis. In this connection, the chemical synthesis of pyridoxine starting from alanine (22) is of interest.

[3] Broquist, H. P., and Snell, E. E., unpublished data.

SUMMARY

Cells of lactic acid bacteria and some other organisms were grown in d fined media variously supplemented with DL-alanine or with vitamin I The vitamin B_6 content of the cells obtained was then determined. Wi lactic acid bacteria, the amount of vitamin B_6 present in the cell was mar edly dependent upon the amount present in the medium. Cells grow with excess of the vitamin contained relatively large amounts of the vit min; cells grown with amounts of the vitamin which limited growth co tained much smaller, but very significant amounts of the vitamin. Som of these organisms grow in a vitamin B_6-free medium when either vit min B_6 or D-alanine is supplied. When such organisms were grown wit D-alanine in place of vitamin B_6, they contained scarcely detectab amounts of the vitamin; amounts much smaller than were present whe the cells were grown with limiting quantities of vitamin B_6. The pr mary rôle of D-alanine in promoting growth of these organisms canno therefore, be as a precursor for synthesis of vitamin B_6.

Some organisms examined synthesized relatively large amounts of vi amin B_6 even though the growth medium contained none of the vitamin The amounts of the vitamin synthesized by these organisms was not ger erally increased by inclusion of D-alanine in the growth medium.

Saccharomyces carlsbergensis 4228, which requires vitamin B_6 for rapi growth, synthesizes some of this vitamin when grown in the presence amounts which limit growth. The amount of vitamin B_6 present in cel of this organism was greatest when it was grown with an excess of pyr doxine, somewhat less when pyridoxal was used, and least when pyrido amine was the form of the vitamin present.

The significance of these results is discussed briefly.

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THE VITAMIN B_6 GROUP

VII. THE RELATION OF D-ALANINE AND VITAMIN B_6 TO GROWTH OF LACTIC ACID BACTERIA*

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Although *Streptococcus faecalis* and *Lactobacillus casei* ordinarily require tamin B_6 for growth, they can be grown in the absence of this vitamin DL-alanine is added to appropriately complete media (2, 3). D-Alanine as shown to be the active component of the DL mixture (3). In at-mpting to explain the nutritional equivalence of vitamin B_6 and D-ala-ne for these organisms, the possibility that D-alanine might serve as a pre-ursor for synthesis of vitamin B_6 was suggested (2). This hypothesis oes not suffice to explain several recent findings, which may be summa-zed as follows: (*a*) Cells of *S. faecalis* grown with DL-alanine replacing itamin B_6 do not produce detectable amounts of codecarboxylase (4) or otransaminase (5), *i.e.* pyridoxal phosphate, although the corresponding poenzymes are produced. (*b*) Direct assay of cells of *S. faecalis* or *L. asei* grown with DL-alanine showed only traces of vitamin B_6 to be resent, in contrast to the much larger amounts of the vitamin present in ells grown with minimal quantities of vitamin B_6 and in the absence of -alanine (1). (*c*) The amount of vitamin B_6 synthesized by a variety of ther microorganisms was not increased by culturing them in the presence f DL-alanine (1). (*d*) Finally, as emphasized in the preceding paper (1), -alanine replaces vitamin B_6 for these organisms only in comparatively omplete media, which contain amino acids and (for *L. casei*) an unidenti-ed substance (3) which are not essential for growth when vitamin B_6 is resent at high levels. D-Alanine is thus not a complete substitute for itamin B_6 under all conditions of growth, but only under specific condi-ions defined in this and preceding papers (1–3).

An alternate explanation for the nutritional equivalence of vitamin B_6 nd D-alanine in certain media would be to assume that D-alanine was it-elf essential for growth, and that vitamin B_6 was required for its synthe-

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sis. In an otherwise complete medium, if D-alanine were supplied formed, vitamin B_6 would no longer be required for growth, or woul required only in much smaller amounts. Data reported below dem strate that D-alanine is present both in cells grown on media contai DL-alanine in the absence of vitamin B_6 and in media containing vita B_6 but no D-alanine. It appears then that D-alanine is neces for growth of the cells, and that it can be synthesized when vitamin B present in the medium, but apparently not in its absence. In the la case, growth can proceed if D-alanine is supplied preformed to the cells.

TABLE I

Conditions for Culture of Various Organisms and Yields of Dry Cells from Variously Supplemented Basal Media

Organism	Bibliographic reference to culture medium used	Incubation		Supplements per liter of ba medium			
				None	DL-Alanine 200 mg.	Pyridoxal 0.001 mg.	P do 1.0
				Dry cell yields, gm. per lit of medium			
		°C.	*hrs.*				
Lactobacillus arabinosus 17-5	(7)	30	24	0.34	0.68	0.58	0.
Leuconostoc mesenteroides 9135	(7)*	30	24	0.26	0.27	0.35	0.
Streptococcus faecalis R	(8)	30	22	0	0.41	0.53	0.
Lactobacillus casei	(7)	37	23	0	0.68	0.54	0.

* 10 mg. of hypoxanthine added per liter of basal medium.

EXPERIMENTAL

Cell Preparations—The methods used for growing sufficient cells assay have been described in the preceding report (1). Additional ganisms used in this investigation and several additional lots of *Strep coccus faecalis* and *Lactobacillus casei* were grown in the same mann Cultural details not described previously are given in Table I. Cells w dried either by lyophilization of aqueous suspensions or by the alcoh ether procedure described by Camien *et al.* (6).

Assay Procedures—Previous work (3) showed that, in the presence o sufficient amount of an enzymatic casein digest, *Lactobacillus casei* gr when either vitamin B_6 (pyridoxal) or D-alanine was added to the mediu L-Alanine and pyruvic acid were essentially inactive. In the absence the enzymatic digest of casein, or in the presence of only limited amour D-alanine was ineffective in promoting growth, although vitamin B_6 still effective. On the basis of these findings and many exploratory s, two media have been devised, in one of which (Medium I, Table growth of *Lactobacillus casei* resulted when samples containing either amin B_6 or D-alanine were added, but in the other of which (Medium Table II) only vitamin B_6 permitted growth. By appropriately treat- samples to remove completely the vitamin B_6, the D-alanine content

TABLE II

Composition of Basal Media

Substance	Amount per 100 ml. double strength medium	
	Medium I	Medium II
	mg.	*mg.*
yptic casein digest	2000	20
id-hydrolyzed casein	1000	1500
paragine	20	20
-Tryptophan	20	20
ystine	40	40
-Alanine		40
extrose	2000	2000
dium acetate*	1200	2400
HPO_4	100	100
H_2PO_4	100	100
$gSO_4 \cdot 7H_2O$	40	40
aCl	2	2
$SO_4 \cdot 7H_2O$	2	2
$nSO_4 \cdot H_2O$	2	2
	γ	γ
iboflavin	80	80
hiamine hydrochloride	40	40
alcium pantothenate	80	80
iacin	80	80
-Aminobenzoic acid	40	40
olic acid	2	2
iotin	0.4	0.4
yridoxal hydrochloride	0.002	

* The amount of sodium acetate indicated is in addition to that furnished by the yptic digest of casein. When prepared as recommended (9), the equivalent of 00 mg. of casein supplies about 930 mg. of sodium acetate.

ould be measured in Medium I, whereas Medium II served as a check to nsure that vitamin B_6 had been completely removed and was thus not ontributing to the response observed in Medium I. The growth esponse to D-alanine in Medium I was more suitable for assay work when mall, suboptimal, amounts of pyridoxal were added to this medium, as ndicated in Table II. The assay procedures used with these media were similar to those customarily employed in the microbiological deter tion of vitamins and amino acids. Samples and water to make 5 ml. autoclaved at 15 pounds pressure for 10 minutes in aluminum-cappe rex test-tubes. The basal medium was autoclaved separately; when it was inoculated and dispensed to the assay tubes, 5 ml. per tube. pyridoxal included in Medium I was added with the inoculum to the viously autoclaved medium. The inoculum culture was grown f hours in Medium I, supplemented with 2 mg. per 10 ml. of DL-ala washed once in saline, resuspended to one-half its original volume, added in the amount of 1 drop for every 5 ml. of basal medium. A

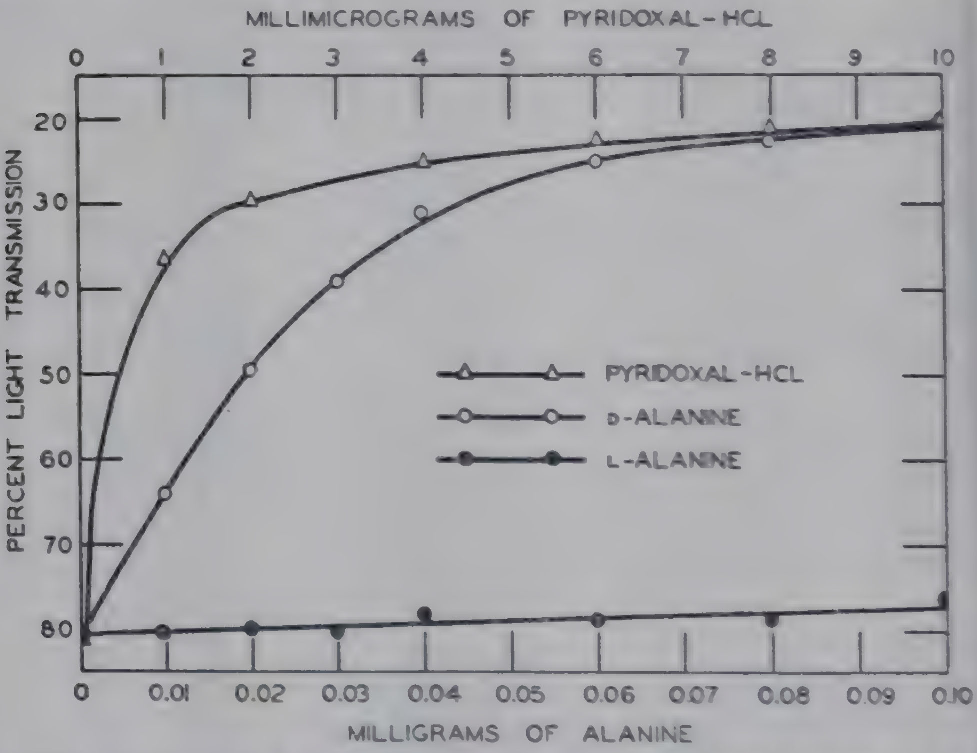
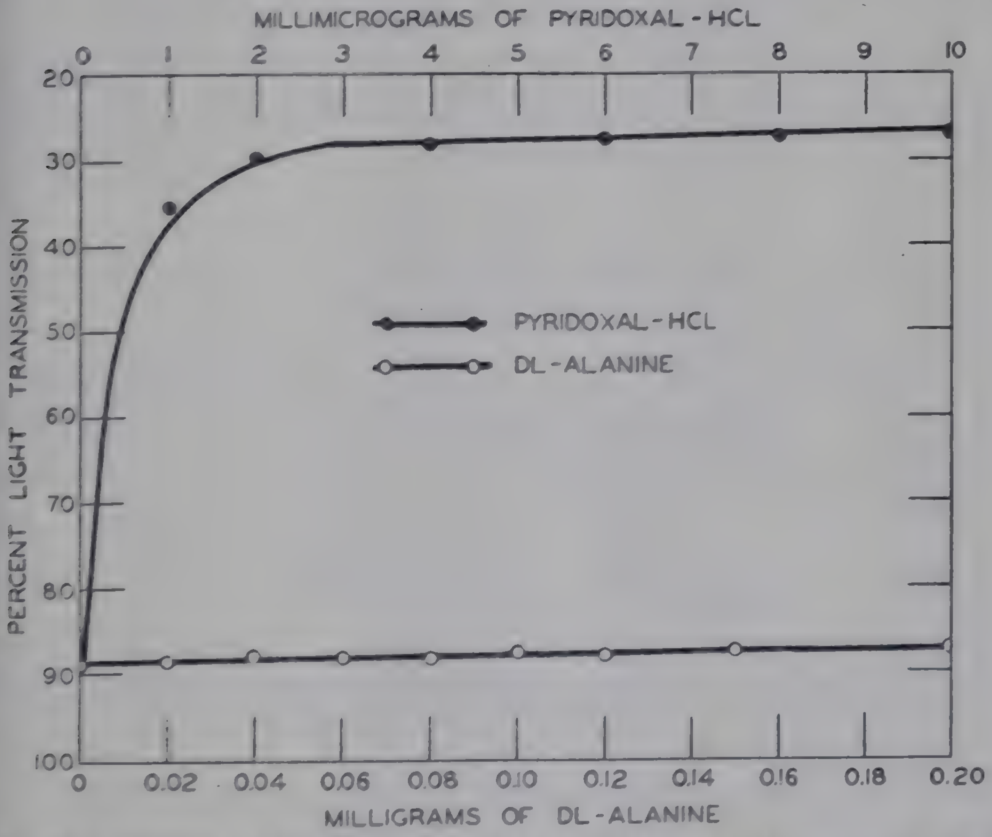


FIG. 1. The response of *Lactobacillus casei* to pyridoxal and to D- and L-a in Medium I, designed for assay of D-alanine.

were incubated for 40 to 45 hours at 37°. The growth response to v addenda was estimated turbidimetrically with the Evelyn photoel colorimeter and the 660 mμ filter.

The comparative growth responses of *Lactobacillus casei* to pyridox isomers of alanine in the two media are shown in Figs. 1 and 2. As cated above, neither isomer of alanine shows growth-promoting ac in Medium II. D-Alanine is highly effective in promoting grow Medium I, whereas L-alanine is ineffective. Pyridoxal is effective in media. When failure to promote growth in Medium II showed sa to be suitably free of vitamin B$_6$, the D-alanine content of samples wa culated from the growth response in Medium I. DL-Alanine was stablish the standard curve in the latter medium. Separate experits confirmed previous observations (3) that D-alanine was twice as ac- on the weight basis as DL-alanine in promoting growth, showing that simultaneous presence of the L isomer neither enhances nor decreases response to D-alanine under these conditions.

reparation of Samples—To prepare samples for assay of D-alanine, two s were essential: (*a*) conversion of any D-alanine present to the free n and (*b*) removal of vitamin B_6, which interferes in the assay. For first purpose, 100 mg. samples of dried cells (or other samples) and 2



IG. 2. The response of *Lactobacillus casei* to DL-alanine and pyridoxal in Medium lesigned to measure completeness of removal of vitamin B_6.

of 3 N hydrochloric acid were sealed in test-tubes, then autoclaved at pounds pressure for 12 to 15 hours. After cooling, the tubes were ned, the hydrolyzed content washed out, the pH adjusted to 6.8 with ssium hydroxide, and the volume adjusted conveniently, usually to nl.

he most convenient treatment found for removing vitamin B_6 was to r the neutralized hydrolysates with suction through short columns of vated charcoal (Darco G-60). By the use of control samples contain- known amounts of vitamin B_6 and of DL-alanine, the amount of char- necessary to remove the vitamin without significantly decreasing the alanine content was readily determined. This amount varied considerably for different lots of the single brand of charcoal tested; hence no constant procedure can be given. For the cell hydrolysates described above, and with an "average" lot of charcoal, a column 2 cm. long and 1.5 cm. in diameter, containing approximately 700 mg. of adsorbent, sufficed to free the sample of vitamin B_6, even with samples containing as much as 40 γ of vitamin B_6 per gm. Aliquots of the filtrates from these samples were diluted appropriately for assay.

The results of assay of several control samples, in which known amounts of pyridoxal and of DL-alanine were added to a casein hydrolysate and assayed for D-alanine, are presented in Table III. Recovery of D-alanine is satisfactory, and illustrates that the mixed amino acids of a protein hydrolysate do not interfere with the determination and that the interfering effects of vitamin B_6 are readily removed by the procedures detailed above. The slightly low recoveries of D-alanine result from removal of small amounts of this substance by charcoal. For purposes of this investigation, *i.e.* to demonstrate whether or not D-alanine occurs in cells of lactic acid bacteria, this result is unimportant.

TABLE III

*Recovery of D-Alanine from Mixtures of DL-Alanine, Pyridoxal, and Casein Hydrolysate**

Added before charcoal treatment		Found after charcoal treatment	
D-Alanine	Pyridoxal hydrochloride	D-Alanine	Pyridoxal hydrochloride
γ per ml.	*mμgm. per ml.*	*γ per ml.*	*mμgm. per ml.*
65.0	200	57.5	0
65.0	200	55.0	0
57.5	200	52.5	0

* The additions were to an acid hydrolysate which contained, per ml., the amino acids derived from 5 mg. of casein. These solutions were then treated with charcoal for removal of vitamin B_6, as described in the text, and diluted for assay.

Assay Results—The results of assays for D-alanine on the four test organisms grown in the variously supplemented media are shown in Table IV. Each of the lactic acid bacteria tested contained from 1 to 2 per cent of D-alanine.[1] Of greatest significance was the finding that cells of *Streptococcus faecalis* and of *Lactobacillus casei* contained roughly equal amounts of D-alanine whether they were grown in the absence of vitamin B_6, under conditions such that they require D-alanine for growth, or whether they were grown without D-alanine in the presence of vitamin B_6, under which conditions D-alanine is not required for growth. Under the latter conditions, the organisms must synthesize this amino acid. *L. arabinosus* and *Leuconostoc mesenteroides*, which grow in the absence of both vitamin B_6 and D-alanine, likewise contain D-alanine in amounts similar to those found in *L. casei* and *Streptococcus faecalis*.

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[1] L-Alanine also is present in such hydrolysates. Assay of hydrolysates of *Streptococcus faecalis* R with *Leuconostoc citrovorum*, which responds equally to D- and L-alanine (10), revealed a total alanine content of 4.6 per cent. Thus D-alanine comprises considerably less than 50 per cent of the total alanine present in this organism.

The validity of the identification of D-alanine as a constituent of these cellular hydrolysates rests to some extent on the specificity of the assay method employed. Of known compounds, only D-alanine and vitamin B_6 are effective in promoting growth, and under the conditions of assay only D-alanine is known to promote growth. The dose-response curves to the samples and to DL-alanine are wholly similar, indicating that D-alanine is the substance present which promotes growth. The results of recovery experiments (Tables III and V) show that D-alanine added to the samples is recovered quantitatively, indicating the precision of the method.

TABLE IV

D-Alanine Content of Cells Cultured in Presence and Absence of D-Alanine or Vitamin B_6

Organism	Supplement to basal medium	Amount per liter	D-Alanine content of dry cells	
			Lot A	Lot B
		mg.	*mg. per gm.*	*mg. per gm.*
Lactobacillus arabinosus......	None		19	13
	DL-Alanine	200	18	16
	Pyridoxal HCl	0.001	19	12
	" "	1.0	21	16
Leuconostoc mesenteroides....	None			8
	DL-Alanine	200		14
	Pyridoxal HCl	0.001		11
	" "	1.0		16
Lactobacillus casei............	DL-Alanine	200	9	11
	Pyridoxal HCl	0.001		5
	" "	1.0	8	8
Streptococcus faecalis.........	DL-Alanine	200		12
	Pyridoxal HCl	0.001		10
	" "	1.0		13

As confirmatory evidence for the presence of D-alanine in such hydrolysates, the method of paper chromatography (11) was employed. A hydrolysate of dried cells of *Lactobacillus casei* from a lot grown with vitamin B_6 (without DL-alanine) was freed so far as possible from hydrochloric acid by vacuum distillation and redissolved in water. 1 ml. of this hydrolysate, equivalent to 20 mg. of dry cells, was streaked across the top of a 46.5 × 57 cm. piece of filter paper (Whatman No. 1). Adjacent to, but separated from, this streak was placed a drop of an aqueous solution containing 5 γ of DL-alanine. After drying, the edge of the strip was immersed in a mixture of 4 parts of pyridine and 1 part of water, the entire assembly being in an enclosed cabinet. After 30 hours development, the paper was removed, dried, and two vertical stripes painted with a solution of ninhydrin in butanol. Exactly corresponding in position on the paper to the control spot due to DL-alanine ($R_F = 0.664$) was a distinct band (one of several) from the hydrolysate. The position of this band being known, the portion not developed with ninhydrin was cut from the paper, leached with water, and the solution concentrated and assayed. The hydrolysate placed on the paper contained 60 γ of D-alanine by direct microbiological assay; the band corresponding in position to pure DL-alanine contained 45 γ of D-alanine by assay.

TABLE V

*Recovery of D-Alanine Added to Solutions of Alkali-Hydrolyzed Casein**

D-Alanine found in hydrolysate	D-Alanine added	Total D-alanine found	D-Alanine recovered	Per cent recovery
γ per ml.	*γ per ml.*	*γ per ml.*	*γ per ml.*	
8.4	8.0	16.5	8.1	101.2
5.4	6.0	11.0	5.6	93.4
7.9	7.5	15.9	8.0	106.7
7.0	7.5	14.7	7.7	103.7
5.7	7.5	13.3	7.6	101.3
5.2	5.0	10.0	4.8	96.0
4.8	5.0	10.0	5.2	104.0

* Hydrolyzed with sodium hydroxide in the autoclave at 121°.

In a subsequent experiment, cells of *Lactobacillus arabinosus* grown i the absence of both vitamin B_6 and D-alanine were hydrolyzed and th hydrolysate chromatographed, as described above. In this instance, 9 per cent of the D-alanine indicated by direct assay was recovered from th band corresponding in position to pure DL-alanine. These results demonstrate unequivocally the presence of this amino acid in hydrolysates o cells of these organisms grown in the absence of DL-alanine, and provid confirming evidence for the validity of the assay procedure employed.

The possibility that the D-alanine found in the hydrolysates arose b racemization during acid hydrolysis was checked by applying the hydrolytic procedure to casein and gelatin in the presence and absence o starch, and to cells of *Torula cremoris*. Assay of these materials indi cated only traces of D-alanine (maximum 0.1 per cent, minimum 0.02 per cent). With these materials, comparatively large amounts of the samples were necessary for assay, thus increasing the difficulty in removing vitamin B_6 and decreasing the specificity of the method. For these reasons, it cannot be said with certainty whether or not any D-alanine was actually present in these hydrolysates. This difficulty in interpretation is not present with hydrolysates of those lactic acid bacteria tested, which contained much greater quantities of D-alanine.

As further evidence that D-alanine did not arise by racemization during hydrolysis, cells (400 mg.) of *Lactobacillus arabinosus* grown in the absence of D-alanine were repeatedly frozen and thawed, then subjected successively to the action of (*a*) autolytic enzymes present in the cells themselves, (*b*) cysteine-activated papain at pH 5.0, and (*c*) calf intestinal mucosa at pH 8.0. Amino nitrogen determinations before and after acid hydrolysis showed liberation of 64 per cent of the total amino nitrogen by this enzymatic treatment. After centrifuging insoluble cell residues, the clarified enzymatic digest was freed of vitamin B_6 by charcoal treatment and assayed. D-Alanine equivalent to 7 mg. per gm. of original cells was found. This was 59 per cent of the value found after acid hydrolysis of a separate portion of the same cells (Table IV, Lot B, grown with 0.001 mg. of pyridoxal hydrochloride per liter). A control sample carried through the same procedure without addition of cells contained no D-alanine. Thus D-alanine is present in the original cells, and does not arise by racemization during acid hydrolysis.

DISCUSSION

The experimental findings which bear upon the rôle of D-alanine in promoting growth of *Streptococcus faecalis* and *Lactobacillus casei* may be summarized as follows: (*a*) In otherwise complete media, growth of these organisms results if *either* D-alanine or vitamin B_6 is added; both substances are not required; (*b*) when the cells are grown with D-alanine and no vitamin B_6, they contain significant quantities of D-alanine but little or no vitamin B_6 (1); (*c*) when the cells are grown with vitamin B_6 but no D-alanine, they contain vitamin B_6 *and* D-alanine, the latter in amounts comparable to cells grown with D-alanine. In the latter case, D-alanine has obviously been synthesized by the cells. In the absence of vitamin B_6, growth does not occur unless D-alanine is supplied preformed, in which case essentially normal growth takes place. It must be concluded from these results that D-alanine, an amino acid of the unnatural series, is essential for growth of these organisms. When vitamin B_6 is available, the organisms synthesize this D-amino acid for themselves; when vitamin B_6 is not available, D-alanine must be supplied preformed. Vitamin B_6

must therefore be essential, either directly or indirectly, for synthe
D-alanine.

A large number of previous investigations has established that ce
D-amino acids may be utilized by a variety of organisms. Certain c
D-amino acids (including D-alanine (12)) have also been shown to
naturally, especially in products of microbiological origin, and D-glut
acid has been reported present in hydrolysates of lactic acid bacteria
Previous to this and related investigations (2, 3), however, no D-a
acid had been recognized as *essential* for growth and metabolic activi
any organism. The finding that D-alanine is essential for certain
acid bacteria is thus unique, and suggests that other amino acids o
D series may also be found essential for certain metabolic reactions.

Limitations of the assay method prevent a decision as to whethe
very small amounts of D-alanine (0.1 per cent) indicated as present i
cells of *Torula cremoris* are actually present. If not, it may mean e
that D-alanine is not generally required by other living organism
that, though required, it is transformed to other products and does
accumulate.

Although the mechanism by which D-alanine is synthesized by *L*
bacillus casei and *Streptococcus faecalis* is unknown, L-alanine is the
logical precursor. Thus L-alanine shows low growth-promoting act
for *Streptococcus faecalis* (though not for *Lactobacillus casei*) in the abs
of vitamin B_6. The fact that vitamin B_6 is required for the synthesis
gests that it may act as a "coracemase" in this process. This sugges
is consistent with the report of Lyman and Kuiken (14) that certain l
acid bacteria showed increased ability to utilize D- in place of L-a
acids when the growth medium was high in vitamin B_6. Similar obse
tions have been made in this laboratory.[2] It is suggestive, in this
nection, that the same type of equilibrium transamination reaction
tween pyridoxal and certain amino acids which has been shown to o
in vitro (15) would also lead to racemization of an optically active a
acid which participated in the reaction.

SUMMARY

This and related investigations (2, 3) have shown that *Lactobac*
casei and *Streptococcus faecalis* grow in appropriate media when e
D-alanine or vitamin B_6 is added. Cells grown with D-alanine do not
thesize significant amounts of vitamin B_6 (1). To clarify the rôl
D-alanine in this phenomenon, a method for its microbiological deter
tion was developed which utilizes *Lactobacillus casei* as the test orga
Since the measured response is given by either D-alanine or vitami

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[2] McCullough, W. G., and Snell, E. E., unpublished.

ples assayed must be freed of the vitamin. This was accomplished charcoal treatment, the completeness of removal of vitamin B_6 being firmed by assay with *Lactobacillus casei* under conditions such that lanine is inactive.

he procedure was then applied to dried cells of *Lactobacillus casei* *Streptococcus faecalis* which had been grown (*a*) without vitamin B_6 with D-alanine present, and (*b*) without D-alanine but with vitamin present. In both cases, D-alanine was found in the cell hydrolysates in nificant quantities. The presence of D-alanine was confirmed by comed use of partition chromatography on paper and bioassay, and, by arate tests it was shown that the D-alanine did not arise by racemizan during hydrolysis. Thus to permit growth these organisms must her be grown under conditions such that they synthesize D-alanine . vitamin B_6 present in an otherwise complete medium), or D-alanine ıst be supplied preformed. Thus it appears that D-alanine is itself esıtial for growth of these organisms, and that vitamin B_6 is involved her directly or indirectly in its synthesis.

Lactobacillus arabinosus and *Leuconostoc mesenteroides*, which grow in edia containing neither D-alanine nor vitamin B_6, also contain D-alanine. ıe amounts of D-alanine found in cells of *Torula cremoris* were so small at its identification by the methods used is not certain.

These results are discussed briefly, and the possibility that vitamin B_6 ay act catalytically in the racemization of certain amino acids is inditted.

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RMATION OF MERCAPTURIC ACIDS BY CYSTINURIC AND NORMAL DOGS

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As a part of a program of investigation of the mechanism of formation
mercapturic acids, it was considered worth while to investigate the
ssibility that the administration of bromobenzene to cystinuric dogs
uld alter the excretion of cystine by the animals. It was found, how-
er, that the excretion of cystine was not altered by the administration
bromobenzene. Since, at the time these experiments were done, choline
d emerged as important in the metabolism of the sulfur-containing
ino acids (1), it was of interest to determine whether or not the ad-
nistration of choline, with and without bromobenzene, would alter the
cretion of cystine. While choline was not found to alter the excretion of
stine profoundly, there were certain observations which led us to in-
stigate this phase in detail with normal dogs. Following the adminis-
ation of choline and bromobenzene, cystine in the urine, as determined
the method of Brand, Cahill, and Kassell (2), was observed to increase
arkedly; there was, however, no significant increase in the excretion of
stine as measured by the method of Sullivan and Hess (3) or by either
ethod after a preliminary precipitation of the cystine as the cuprous
ercaptide. Since the cystinuric dogs were males and since the excretion
cystine interfered with the determination of the "unknown disulfide,"
rther studies were made with normal dogs; the majority of the studies
ere made with trained females so that catheterization could be employed
the collection of the urine. It was found that the optimal formation
mercapturic acid was dependent upon a supply of extra choline in the
iet as well as upon a supply of cystine or precursors of cystine. As the
oline was given, the excretion of disulfides, as measured by iodometric
tration (4), was found to increase markedly. The urines were found to
ve a positive nitroprusside reaction and to respond to the test of Brand,
ahill, and Kassell for disulfides but were negative to the test of Sullivan
nd Hess for cystine. The compound responsible for the disulfide reac-
ons was isolated in small quantities, but its identity was not established.

In the course of these studies a relatively simple colorimetric method for
e estimation of *p*-bromophenylmercapturic acid was developed.

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EXPERIMENTAL

Colorimetric Estimation of p-Bromophenylmercapturic Acid

It was found that the excretion of the disulfide material interfered the determination of the mercapturic acid by the method of Steko the blanks were erroneously high and, in several cases, considerably mercapturic acid could be isolated from the urine than was indicate the determination. In order to avoid these difficulties, a conver colorimetric method was developed. The method was essentially adaptation of the method of Brand, Cahill, and Kassell (2) to the est tion of the *p*-bromothiophenol formed in the alkaline cleavag the *p*-bromophenylmercapturic acid. The particular disadvantage of method is that the procedure must be completed without delay as as the alkaline cleavage is complete.

TABLE I

Recoveries of p-Bromophenylmercapturic Acid from Solution

Recoveries are given in the units of the Klett-Summerson colorimeter. Cy was dissolved in acid and diluted to volume with the 5 per cent solution of urea

Mercapturic acid added	Recovery	Cystine standard		Ratio, mercap acid to cysti
mg.	*colorimeter units*	*mg.*	*colorimeter units*	
0	14.5	0.1	39.0	0.37
0.2	29.0	0.2	77.1	0.38
0.5	73.0	0.5	191.3	0.38
1.0	143.5			0.38*

* Calculated.

Procedure—5 ml. of urine (containing less than 5 mg. of mercapturic a were placed in a 50 ml. Erlenmeyer flask and 2.5 ml. of 2.5 N sodium droxide were added. A watch-glass was placed over the mouth of flask and the solution was boiled gently on a hot-plate for 10 minu The flask was then cooled to room temperature and the contents were ri into a 25 ml. volumetric flask with a 5 per cent solution of urea; 2.5 of 2.5 N acetic acid were added and the contents were diluted to vol with the 5 per cent solution of urea. Aliquots of this solution were in the determination which was carried out as described by Brand, Ca and Kassell, except that a 12 minute period of color development found to be more satisfactory than the 8 minute period.

Recovery of p-Bromophenylmercapturic Acid—Some recoveries of bromophenylmercapturic acid are illustrated in Table I. The recove are given as units of the Klett-Summerson colorimeter with Filter is demonstrated in Table I, it was possible to use solutions of cystine the standard in the determination. Obviously, the treatment with ali was omitted with the solutions of cystine. In Table II, recoveries the mercapturic acid added to dog and rat urines are given. It is seen it good recoveries were obtained. In Table III, the interferences of stine, cysteine, methionine, and of a concentrate of urine are shown. All

TABLE II

Recoveries of p-Bromophenylmercapturic Acid Added to Dog and Rat Urines

The mercapturic acid was dissolved in 100 ml. of urine and 5 ml. aliquots, con- ning the amounts indicated, were taken for analysis as described in the text. stine was used as a standard.

Mercapturic acid	Dog urine	Rat urine
mg.	*mg.*	*mg.*
0.10	0.09	0.11
0.50	0.51	0.55
1.00	1.04	1.09

TABLE III

Interference of Sulfur-Containing Compounds on Determination of p-Bromophenylmercapturic Acid

Only one concentration of mercapturic acid (0.2 mg. in 5 ml.) was used. All com- unds were made up in pooled dog urine. Cystine was used as a standard.

Compound added	Amount	Recovery of mercapturic acid
	mg.	*per cent*
ystine	0.1	101
“	0.2	105
“	0.5	112
ysteine HCl	0.1	98
“ “	0.2	102
“ “	0.5	106
lethionine	0.1	105
“	0.2	107
“	0.5	112
	gm. per 5 ml.	
rine concentrate	1	110

ese materials interfered somewhat, but at ordinary concentrations the terferences were negligible.

Formation of Mercapturic Acid by Cystinuric Dogs

Cystine was determined by the methods of Sullivan and Hess (3) and f Brand, Cahill, and Kassell (2). The method described by Brand, Cahill, and Kassell was used for the precipitation of cystine as the cuprous captide. The dogs used in this study were two male Irish terrier tained through the courtesy of Dr. Erwin Brand. While partitions o urinary sulfur were made on several specimens of urine, the results not especially informative and are not given here. Mercapturic acid determined by the method described above. The basal diet for t studies and for the studies with the normal dogs had the following position: 25 parts of crude casein, 45 parts of sucrose, 20 parts of lar parts of powdered agar, 3 parts of Cowgill's salt mixture (6), 3 part dried brewers' yeast, and 2 parts of cod liver oil. Dog 2X, weighing kilos, was given 220 gm. of the diet daily; Dog 5X weighed 10.8 kilos was given 170 gm. daily. Choline chloride was given in capsules at

TABLE IV

Effect of Choline on Excretion of Cystine by Cystinuric Dogs

Cystine was determined by the methods of Sullivan and Hess and of Brand, Ca and Kassell directly on the urine. Each period represented a 3 day collectio urine. The results are given in mg. of cystine excreted per day.

Period No.	Dog 2X		Dog 5X		Supplement
	Brand, Cahill, and Kassell	Sullivan and Hess	Brand, Cahill, and Kassell	Sullivan and Hess	
I	265	123	95	73	None
II	270	121	92	73	"
III	245	123	100	78	"
IV	290	135	95	98	Choline
V	290	149	105	100	"
VI	215	165	85	88	"

rate of 1 gm. per day and bromobenzene was given at the rate of 2 per day.

One of the first studies is summarized in Table IV. There were points of interest in this study. First, the contrast between the two me ods for cystine was unexpected; with Dog 2X very poor agreement observed, while with Dog 5X rather good agreement was observ Secondly, the administration of extra choline was followed by a slight crease in the excretion of cystine, as measured by the method of Sulli and Hess, but there was no increase as measured by the method of Bra Cahill, and Kassell. In view of the later studies, the increase in ex tion of cystine as measured by the method of Sullivan and Hess was considered as significant. Excellent agreement between the two meth was obtained, however, for the urine of Dog 2X when the methods inv ing the preliminary precipitation of cystine as the cuprous mercaptide ed. For example, the urine of Period III (Table IV) was found ntain 129 mg. of cystine by the method of Brand, Cahill, and Kassell 126 mg. by the method of Sullivan and Hess, utilizing, in both cases, ninary precipitation of the cystine as the cuprous mercaptide, whereas he direct methods the values were 245 mg. and 123 mg. respectively. idies of the excretion of cystine and mercapturic acid following the nistration of bromobenzene and choline are summarized in Table It is evident that the excretion of cystine was not altered by the adstration of bromobenzene. It is apparent that some compound other cystine, presumed to be a disulfide, was excreted when choline and mobenzene were given to the cystinuric dogs; this compound responded he test for disulfides of Brand, Cahill, and Kassell, but it was not pretated as a cuprous mercaptide under conditions leading to quantita- precipitation of cystine.

Table V

Excretion of Mercapturic Acid and Cystine by Cystinuric Dog Receiving Bromobenzene

ystine was determined by the method of Brand, Cahill, and Kassell, utilizing recipitation of cystine as the cuprous mercaptide as well as by the direct method. data presented were those for Dog 5X, which was given 2 gm. of bromobenzene 1 gm. of choline chloride daily as indicated.

iod No.	Cystine excretion, direct method	Cystine excretion, cuprous mercaptide method	Mercapturic acid excretion	Supplement		
	mg. per day	*mg. per day*	*mg. per day*			
1		112		Basal		
2		97		"		
3		97		"		
4	84	84		Choline		
5	105	106		"		
6	97	94		"		
7	249	104	1210	"	and	bromobenzene
8	333	106	1470	"	"	"
9	381	96	1580	"	"	"

Studies with Normal Dogs

Iercapturic acid was determined as described above. The disulfide tent of the urine was determined by the method of Virtue and Lewis in a zinc hydroxide filtrate of the urine. Bromobenzene, choline oride, cystine, and homocystine were given in gelatin capsules.

n one of the first experiments the excretion of mercapturic acid by a le dog was determined by isolation and purification of the mercapturic acid from 3 day collections of urine. The urines were treated with a amount of Darco and were filtered. The filtered urines were then acid to Congo red by the addition of concentrated hydrochloric ac were heated on the steam bath for ½ hour. The cooled urine was in a continuous extractor and extracted for 24 hours with chloroform. chloroform solution was evaporated to dryness and the residu dissolved in dilute bicarbonate solution. The bicarbonate solutio extracted with two portions of ethyl acetate which were discarded. bicarbonate solution was acidified with hydrochloric acid and w lowed to stand in the ice box 3 days. The mercapturic acid whic crystallized was collected on a sintered glass filter, dried at 100° hours, and weighed. The results are given in Table VI. It is app

Table VI

Effect of Choline on Formation of Mercapturic Acid

The mercapturic acid was isolated as described in the text. The dog rece gm. of bromobenzene daily and was given 1 gm. of choline chloride daily as indi

Days	Mercapturic acid isolated	Nitroprusside test	Supplement
	gm.		
1–3	2.7	Negative	None
4–6	4.1	3+	Choline chlorid
7–9	2.6	+	None
10–12	4.6	3+	Choline chlorid
13–15	3.8	2+	None

that the administration of choline chloride was followed by an increa the excretion of mercapturic acid.

Several female dogs were used in further studies in which the excr of mercapturic acid and of disulfide was determined. The most str of these studies was with a trained female which was given 2 gm. of br benzene daily for over 90 days. The dog consumed each day's food no weight during the study, and was never in distress except when choline was omitted from the diet. A portion of the study is giv Table VII. The disulfide value of 0.5 mM was the value ordinarily f with a "nitroprusside-negative" urine. The nitroprusside test be strongly positive at values near 1 mM per day. It is seen that a signif excretion of disulfide did not begin until choline was given and this ro paralleled the increase in excretion of mercapturic acid. When ch was given, the excretion of mercapturic acid was increased about 50 pe and decreased to nearly the basal level when the administration of ch was ended.

e of the dogs used in the study, a female hound, refused all food as as bromobenzene was given and lost weight very rapidly during the y. It was possible with this animal to demonstrate a marked effect wing the administration of choline; the data are summarized in Table . At the conclusion of the experiment the animal was weak and in

TABLE VII

Effect of Choline on Excretion of Mercapturic Acid

female dog, weight 10.5 kilos, receiving 200 gm. of the casein diet, was given 2.0 of bromobenzene and 2.0 gm. of L-cystine daily. Choline chloride was given at ate of 1 gm. per day. The total urine specimens were obtained by catheteri- n.

Days	Mercapturic acid excretion	Disulfide excretion	Supplement
	mM S	*mM S*	
1	3.5	0.3	None
2	3.8	0.5	"
3	4.0	0.5	"
10	3.9	0.5	"
11	4.1	0.6	"
12	4.1	0.5	"
13	4.7	1.3	Choline
14	6.3	2.7	"
15	6.8	2.8	"
16	6.5	2.8	"
17	6.5	2.9	"
18	6.4	2.4	None
19	5.5	2.1	"
20	5.3	1.8	"
21	5.2	1.9	"
22	4.9	1.9	"
23	4.9	1.9	Choline
24	6.3	2.3	"
25	6.2	2.6	"
26	6.6	2.6	"
27	6.4	2.9	"

y poor condition, but, nevertheless, there was a marked effect of the ministration of choline on the excretion of mercapturic acid.

Stekol (7) has reported that homocystine would replace cystine in the owth of rats receiving bromobenzene but would not increase the ex- etion of mercapturic acid. These findings were checked with the dog d the results are summarized in Table IX. It is obvious that homo- stine replaced the cystine in the formation of mercapturic acid.

Characterization of "Disulfide Compound"

The evidence for the disulfide nature of the compound may be marized as follows: a positive nitroprusside test was obtained afte

Table VIII

Effect of Choline on Excretion of Mercapturic Acid by Fasting Dog

The initial weight of the dog was 10.9 kilos and the final weight was 8.9 ki gm. of bromobenzene and 2 gm. of L-cystine were administered daily.

Days	Mercapturic acid excretion	Disulfide excretion	Supplemen
	mM S	*mM S*	
1	3.9	1.0	None
2	4.1	1.1	"
3	4.2	1.2	"
4	2.8	1.2	"
5	4.8	2.9	Choline chlor
6	5.0	3.4	" "
7	6.3	3.0	" "
8	5.2	2.9	" "
9	3.2	2.4	" "

Table IX

Replacement of Cystine by Homocystine in Formation of Mercapturic Acid

The dog of Table VII was used in this study. 2 mg. of bromobenzene and of choline chloride were given daily. 2.4 gm. of DL-homocystine were given as cated.

Days	Mercapturic acid excretion	Disulfide excretion	Supplement
	mM S	*mM S*	
1	6.5	3.5	Homocystine
2	6.7	4.1	"
3	7.1	3.8	"
4	6.4	3.6	None
5	4.2	2.3	"
6	4.4	2.4	Homocystine
7	6.4	3.4	"
8	6.5	3.3	"
9	6.6	3.3	"
10	6.4	3.1	"
11	5.0	1.9	None
12	4.1	1.6	"

not before, treatment with sodium cyanide or reduction with metallic in dilute acid; the reaction of Brand *et al.* was strongly positive, alth full color development was not obtained until after 1 hour; and al

ve reactions were destroyed by a preliminary oxidation of the samples
h bromine water.

he compound did not appear to be a simple derivative of cystine,
e the reaction of Sullivan and Hess was negative before and after hy-
lysis with hydrochloric acid, after aging of the urines, or after treatment
concentrate with sodium in liquid ammonia. The material did not
n a cuprous mercaptide.

concentrate of the compound was obtained in the following manner:
e pooled urines were concentrated to dryness *in vacuo* and at low tem-
ature and the dry residue was extracted with hot methanol. The
thanol extract was then passed through a column containing activated
mina; the compound was adsorbed and could be eluted with a mixture
90 per cent methanol and 10 per cent ammonium hydroxide. A hy-
scopic material was obtained by the addition of acetone to a solution of
material in methanol. This material has a sulfur content of about 15
cent but appeared to be impure. A crystalline picrate with a sulfur
tent of 8.1 per cent was obtained from the treatment of the methanol
ution with picric acid. This material melted at 221° and there was
narked depression of the melting point when the picrate was mixed with
picrate prepared from cystine betaine.

The compound was labile and almost any manipulation was accom-
nied by losses of the material. When a solution of the material was
iled, a strong garlic-like odor was observed. The volatile material
rmed a derivative with mercuric chloride but was not identified.

Under the influence of an electrical current, the compound migrated
pidly toward the cathode; the migration was reversed by the addition
hydrochloric acid to the compartment in which the compound had ac-
mulated. The compound, therefore, was amphoteric.

DISCUSSION

If the cystine utilized in the formation of mercapturic acids were de-
ved from the same immediate metabolic source as the cystine excreted
the cystinuric dog, it would be expected that the administration of
omobenzene would serve to depress the excretion of cystine. Since the
lministration of bromobenzene (and the subsequent formation of mer-
pturic acid) was without effect on the excretion of cystine by the cys-
nuric dog, it may be concluded that different metabolic pathways are
volved. The simplest explanation would appear to be that the forma-
on of mercapturic acid is a function of the liver and that the fundamental
ror of metabolism leading to the excretion of cystine is to be found in
e kidney.

The excretion of a compound or compounds responding to tests char-

acteristic of disulfide compounds by the cystinuric and normal do
ceiving bromobenzene and choline is a phenomenon which requires fu
investigation. Whether or not the excretion of the material is conn
metabolically with the formation of mercapturic acids cannot be ans
from the information available at this time. The effect of choli
bringing about an increase in the excretion of mercapturic acid
appear to have several possible explanations; the more plausible of
would appear to be that the effects of choline are non-specific in natur
are a reflection of the participation of choline in the preservation
patic integrity in the presence of toxic agents. Whether or not disu
are excreted when other toxic agents are administered with cholin
not been determined.

The author is indebted to Professor V. du Vigneaud for his interest
his suggestions during the course of these studies.

SUMMARY

It has been demonstrated that the administration of bromobenzen
cystinuric dogs did not decrease the excretion of cystine by the ani
The administration of choline with the bromobenzene was followe
the excretion of a compound or compounds which responded to tests
sidered to be characteristic of the disulfide grouping. Normal dogs
found to excrete the same or similar compounds when choline and br
benzene were administered; the administration of the choline with
bromobenzene appeared to increase the excretion of mercapturic aci

A colorimetric method suitable for the estimation of *p*-bromophenyl
capturic acid in the urine of dogs was developed.

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THE SOURCE OF SULFATE IN THE FORMATION OF ETHEREAL SULFATES

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aidlaw and Young, in a recent report (1), have investigated the ques- of whether or not inorganic sulfate is utilized in the synthesis of ethe- sulfates. These investigators have shown, beyond any reasonable bt, that inorganic sulfate may be utilized in the synthesis, but their , as reported, do not permit the conclusion that inorganic sulfate of the is utilized to any appreciable extent in this synthesis. Several years , in the course of an investigation of growth and mercapturic acid nation, we were interested in ascertaining whether or not inorganic ate in the diet would facilitate the growth of animals receiving a phenol he diet. We had obtained results from the feeding of bromobenzene ch appeared to be consistent with a concept that as long as the dietary fur (cystine and methionine) was adequate, there was a constant excre- of total detoxication products (ethereal sulfates plus mercapturic ds). Dietary changes, such as the addition of extra choline (2), served increase the excretion of mercapturic acids, but this increase of excre- of mercapturic acids appeared to be at the expense of the excretion ethereal sulfates. It became of some importance, therefore, to deter- ne whether the addition of inorganic sulfate to the diet would bring out a resumption of growth of rats receiving amounts of phenol sufficient retard growth. Since bromobenzene was used in the studies of the mation of mercapturic acids, the related phenol, *p*-bromophenol, was d in the study of formation of ethereal sulfates. A relatively high el of *p*-bromophenol was necessary to bring about the cessation of wth of animals on the diet used in these studies. When growth had en stopped by the administration of *p*-bromophenol, it was found that rganic sulfate was not effective in bringing about a resumption of growth, ereas cystine was very effective. In order to demonstrate that the ects on growth were correlated with the formation of ethereal sulfates tain of the animals were placed in cages permitting quantitative collec- n of urine, and the excretion of ethereal sulfates was determined; it was und that the addition of inorganic sulfate to the diet was ineffective in the facilitation of the formation of ethereal sulfates, but that the ad of cystine was quite effective. Thus, the resumption of growth, up addition of cystine, was correlated with an increase in the excre ethereal sulfates.

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It may be concluded from the data presented here that, in so far as d sources of sulfur are concerned, it is of little importance whether a pound is excreted as a mercapturic acid or as an ethereal sulfate demands upon the dietary sulfur are identical. It must be empha however, that formation of ethereal sulfates does not appear to be a ligatory process; doubling the amount of phenol in the diet did not the growth of the animals and did not bring about a proportiona crease in the excretion of ethereal sulfates.

TABLE I

Sulfur Partitions of Urines of Rats Receiving Bromobenzene

For description, see the text. The results are expressed in mg. per day.

Diet	Inorganic sulfate sulfur	Organic sulfur	Ethereal sulfate sulfur	Mercapturi sulfu
Basal	13.9 ± 2.4	6.1 ± 3.1	2.2 ± 1.4	
Cystine	31.2 ± 4.6	23.3 ± 4.7	2.5 ± 1.4	
Bromobenzene, cystine	24.0 ± 5.1	24.6 ± 4.4	7.1 ± 3.8	11.3 ±
Bromobenzene, cystine, choline	22.6 ± 6.4	25.1 ± 3.5	5.8 ± 1.8	15.3 ±

EXPERIMENTAL

Total Detoxication of Bromobenzene—In Table I a study of the e tion of sulfur by adult male rats (250 to 300 gm.) receiving bromoben is summarized. These animals were given a diet containing casein 8 p dextrin 41 parts, sucrose 15 parts, Mazola oil 30 parts, salt mixtur 4 parts, and agar 2 parts. The water- and fat-soluble vitamins were plied as described by Chandler and du Vigneaud (4). Bromobenzene incorporated at the level of 1 ml. per 100 gm. of diet, and cystine given as 1 per cent of the total diet. Extra choline was given at the of 50 mg. daily. Food consumption was limited to 7 gm. daily and co tions of urine were made in 5 day periods. The methods of Folin (5) of Benedict (6) were used in the determination of the sulfur in the u The method of Binkley (2) was used for the determination of mercap acid. The data recorded in Table I represent the averages for s animals with each animal on each diet for 3 or more 5 day periods. variations indicated by plus and minus figures are the maximum d is from the averages; the average deviations were about one-third he maximum deviations.

n agreement with the results obtained with dogs (2), it was found that administration of choline was followed by an increase of the excretion nercapturic acids; the total excretion of detoxication products was not atly influenced and much of the increase in the excretion of mercapturic d was at the expense of the ethereal sulfates.

Relationship of Level of Bromobenzene and Cystine to Growth of White ts—In an attempt to assess to what extent the formation of mercapturic ds was an obligatory process, a large number of experiments were made

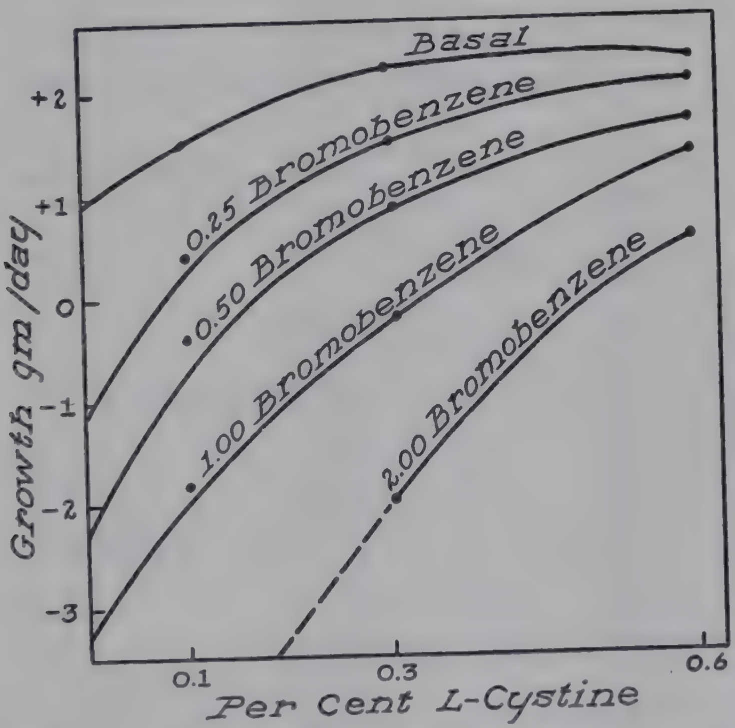

FIG. 1. Growth of rats receiving various levels of bromobenzene and cystine in ne diet.

n the growth of white rats receiving bromobenzene. The casein diet, escribed above, was used in these studies. Animals weighing between 0 and 70 gm. were used in these studies and only the data of those animals which could be induced to consume 5 gm. of diet per day are included. Each point represents the average growth data of six or more animals or the initial 10 day period after the addition of bromobenzene and extra ystine to the diet; the large number of animals involved in the study precluded the use of longer periods. These "titration" studies are summarized n Fig. 1. The animals, which received 2 per cent of their diet as bromobenzene, did not consume sufficient food when the concentration of cystine was below 0.3 per cent to give valid data. Otherwise, each concentration of bromobenzene was tested at levels of 0.0, 0.1, 0.3, and 0.6 per extra cystine in the diet. The addition of the larger amounts of br benzene to the diet was accompanied by loss of weight, but, even so, t was a relationship between rate of loss of weight and the concentrati bromobenzene.

Growth of Rats Receiving p-Bromophenol—In Table II, the growth food consumption of rats receiving *p*-bromophenol are illustrated. T animals received the basal diet described above. While several la litters were used with the same results, these are the animals used in study of excretion of ethereal sulfates reported in the next section.

TABLE II

Growth and Food Consumption of Rats Receiving p-Bromophenol

Rat No.	Days	Food consumption	Growth	Supplement to basal diet	
		gm. per day	*gm. per day*		
CJ606	0–70	4.5	0.8	None	
CJ607	0–10	4.1	0.5	"	
	10–20	3.7	0.1	1% *p*-bromophenol	
	20–30	3.7	0.0	2% "	
	30–70	5.4	1.2	2% "	+ 0.8% cystine
CJ608	0–10	4.5	0.5	None	
	10–20	2.8	−0.1	1% *p*-bromophenol	
	20–50	4.1	0.0	2% "	
	50–70	5.3	−0.1	2% "	+ 0.5% Na_2SO_4
CJ609	0–10	4.5	0.4	None	
	10–20	3.2	0.0	1% *p*-bromophenol	
	20–30	3.8	0.1	2% "	
	30–40	6.1	1.9	2% "	+ 0.8% cystine
	40–70	5.5	−0.3	2% "	+ 0.5% Na_2SO_4

seen that, in contrast to the results noted with bromobenzene, doubl the level of *p*-bromophenol does not result in any greater loss of weig and the animals maintained their weight for long periods of time while ceiving as much as 2 per cent of their diet as *p*-bromophenol. When c tine, but not inorganic sulfate, was added to the diet, there was an imm ate resumption of growth. A slight gain of weight, noted immediately a the addition of sodium sulfate to the diet, appeared to be correlated w an increased consumption of water; there was, however, no diarrhea this concentration of sodium sulfate.

Excretion of Ethereal Sulfates by Rats Receiving p-Bromophenol— data summarized in Table III include the results on the animals of Ta nd of additional groups; therefore each figure is the average of four or e animals and a minimum of five 10 day periods is included in each age. It is obvious from these data that, although there is a slight in- se in the formation of ethereal sulfates following the administration organic sulfate, this increase is in no sense of the order of magnitude he increase noted after the addition of cystine to the diet. The in- se in consumption of the diet (older animals in these periods) would ount for the increase in excretion of ethereal sulfates following the ad- istration of inorganic sulfate. The doubling of the level of *p*-bromo- nol was not followed by an increase in the excretion of ethereal sulfates , as noted above, was there any further effect on growth. Thus, the nation of ethereal sulfates does not appear to be an obligatory process he same sense as the formation of mercapturic acids (Fig. 1).

TABLE III

Sulfate Partition of Urines of Rats Receiving p-Bromophenol

Diet	Food consumption	Growth	Inorganic sulfate	Ethereal sulfate
	gm. per day	*gm. per day*	*mg. Na_2SO_4 per day*	*mg. Na_2SO_4 per day*
sal	4.5	0.8	30.1	8.0
phenol	4.2	0.1	10.0	32.5
"	4.0	0.1	12.7	32.5
" + 0.5% Na_2SO_4	5.3	0.1	271.6	41.7
" + 0.8% cystine	5.6	1.7	249.5	90.8

DISCUSSION

The apparent inconsistency between our observations and those made llowing the administration of radioactive sodium sulfate may be ex- icable on the basis of the amounts and route of administration of the lfate. Since inorganic sulfate is either a non-threshold substance or is substance with a very low threshold, it is cleared rapidly by the kidney d it is probable that no significant increase in the level of sulfate in e tissues is ever attained by the feeding of sodium sulfate in the diet. n the other hand, the single injection of sulfate, coincident with the ad- inistration of the phenol, would provide a more advantageous situation r conjugation. It is likewise possible that small amounts of inorganic lfate may be distributed in the tissues without undue loss by renal mech- nisms and thus enter into ethereal sulfate formation even though no crease in such formation may have occurred.

From the observations of Laidlaw and Young it may be said that ad- inistered inorganic sulfate may be found in the ethereal sulfates formed

by the animal. From our data it may be stated that administered
ganic sulfate is ineffective in bringing about an increase in the excr
of ethereal sulfates or the resumption of growth of rats receiving p
mophenol. As explained above, these conclusions are not inconsis
In any event, the basic question of whether or not inorganic sulfa
esterified directly with a phenol in the formation of ethereal sulfates
not been answered and must await results of studies with isolated syst
Whatever the exact mechanism of formation of ethereal sulfates ma
it is obvious that, in so far as dietary sulfur is concerned, inorganic su
is ineffective and the formation of ethereal sulfates, analogous to the fo
tion of mercapturic acids, places demands upon the sulfur-contai
amino acids of the diet.

The author is indebted to Professor V. du Vigneaud for his interest
suggestions during the course of this study.

SUMMARY

It has been demonstrated that inorganic sulfate in the diet was ine
tive in the production of a resumption of growth of rats receiving p-bro
phenol. Cystine was found to be effective. The effects on growth
correlated, in the case of inorganic sulfate, with no increase of the excre
of ethereal sulfates and, in the case of cystine, with a significant incr
of the excretion of ethereal sulfates.

Certain data concerning the relationship between the formation of
capturic acids and ethereal sulfates have been included in this report.

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THE INFLUENCE OF OVARIECTOMY ON THE CHEMICAL COMPOSITION OF REGENERATING RAT LIVER*

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Because of the remarkable rate of restoration of liver tissue, the par-
lly hepatectomized rat has been recognized as a useful preparation in
ich to study the process of active tissue growth (1–13) and the par-
ipation of various cellular components in this process (*cf.* (14)). How-
er, the very early postoperative period, during which extreme fluctua-
ns in liver constituents occur, has been almost uniformly neglected.
rallel studies of the various liver components have not been made in
ch a manner as to permit integration of the rates of restoration of lip-
e, protein, and water during early hepatic regeneration.

In the present investigation, the nitrogen, water, and lipide content of
e regenerating rat liver has been followed at frequent intervals during
e first few days after partial hepatectomy. The results obtained have
een correlated with the increase in size of the remaining liver and with
e onset of true growth as indicated by mitotic activity. In addition,
e rôle of ovarian secretions in the mobilization of these liver constit-
ents has been investigated.

Methods

Adult female rats of an inbred Sherman strain were partially hepatec-
omized when they were about 150 gm. in weight. The operative pro-
edure, modified after that of Higgins and Anderson (1), has been de-
cribed in detail (15). One group of animals had been subjected to
ilateral ovariectomy 16 days earlier. The amount of liver removed
om an earlier series of twenty-three ovariectomized animals averaged 65
er cent, with a standard deviation of 2.7 per cent (15).

From the median and left lateral lobes of that portion of the liver re-

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* A preliminary report of the data has been presented (*Federation Proc.*, 7, 122
948)).

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os Angeles 24.

moved at operation, samples were obtained for water, total nitrogen, total lipide determinations, as well as for histological examination. animals were individually caged, allowed free access to Purina labora chow and water, and were sacrificed at intervals of from 4 hours t days following partial hepatectomy. At autopsy, the amount of l present was determined, and samples were again taken for analysis.

Determination of liver water was carried out on an aliquot of the fr tissue, weighed rapidly in a small tared vial on the analytical bala The tissue was then dried overnight at 105° to constant weight, and difference between wet and dry weights taken as the water content. S able aliquots of liver tissue were analyzed for nitrogen by the semimic Kjeldahl procedure. Titration of the ammonia distillate was perform by the method of Sobel *et al.* (16), with the exception that a mixture methyl red and brom-cresol green was used as the indicator. To nitrogen multiplied by the factor 6.25 was used to estimate the amount protein present. It is recognized, however, that this method of calcu tion does not give unequivocal values for protein. Total lipide was me ured by a modification of the gravimetric procedure described by Robe and Samuels (17). This method included exhaustive Soxhlet extract of the ethanol-dehydrated, ground sample with 95 per cent ethanol a diethyl ether successively, followed by resolution of the dried extract w petroleum ether.

Results

Fig. 1 reveals that partial hepatectomy resulted in a transitory loss weight in both control and ovariectomized rats. The rate of loss, as w as the later return to normal, was essentially the same in both groups animals. This early loss of weight following partial removal of the li approximated 10 per cent of the preoperative body weight, and was pr ably due to the initial drop in food consumption noted by other work (*cf.* (1, 3, 18)). In Fig. 1 the rate of restoration of liver mass in b groups is also shown. The per cent of normal liver present in each anir at the time of autopsy was estimated from the amount of liver that wo be expected to be present in a non-hepatectomized rat of the same bo weight. It will be noted that the rate of restoration of liver mass mediately after partial hepatectomy in the ovariectomized group appea to lag behind that in the control group. This lag was probably not nificant, however, and, at any rate, was overcome within 10 to 12 hou After this initial period, both groups of rats exhibited a rapid spurt in li growth between 20 to 30 hours after operation. At this time, the ma mum loss in body weight was observed.

Analysis of samples of liver removed at operation and at autopsy ted an evaluation of the changes in the various liver constituents as oration progressed. Figs. 2, *A* and 2, *B* show the rate of increase of l liver protein, lipide, and water per 100 gm. of body weight in control ovariectomized animals, respectively. In both groups of animals a y was observed in the accumulation of protein and water for 16 to 24 rs after partial hepatectomy. During the same period of time, how-

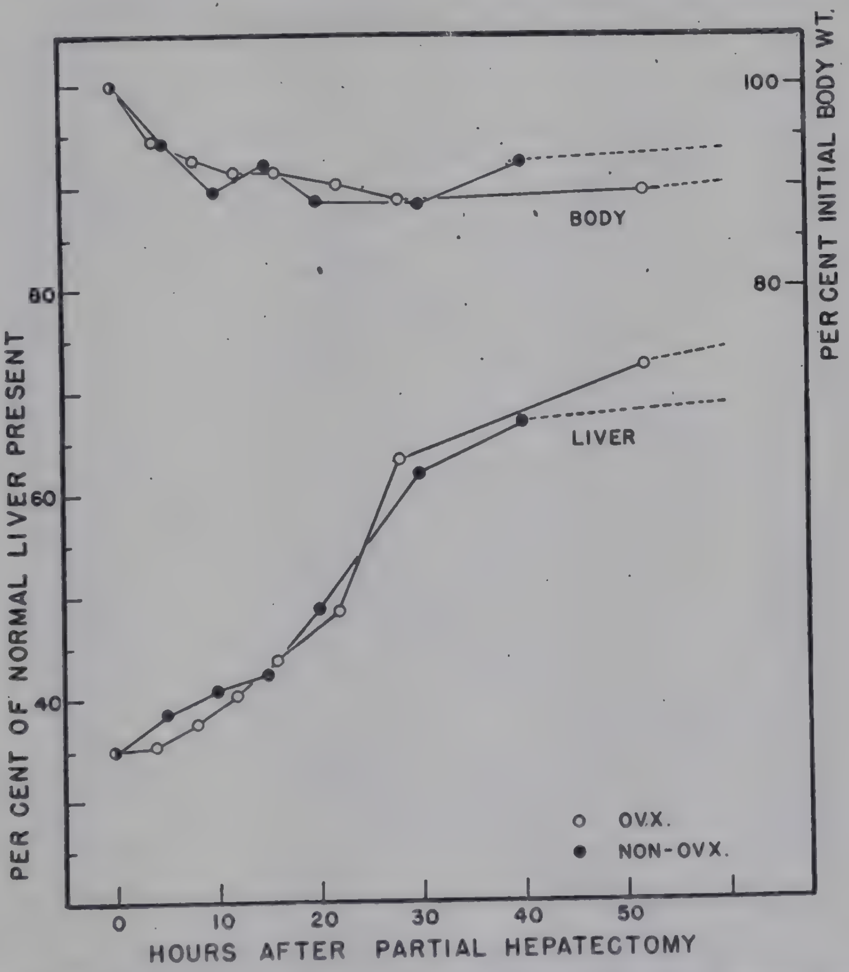

FIG. 1. The rate of restoration of liver and body weight after partial hepatectomy. ch point on this and subsequent curves represents the average of approximately animals, except for the initial values (at 0 hour) which represent from six to irty-two animals. The dotted lines in this and following figures show the real pes of the curves, based on values obtained at later times after operation (3 to days). The latter values are not shown because of the emphasis on the early riod of liver restoration.

er, lipide deposition occurred at a rapid rate in the non-ovariectomized oup (Fig. 2, *A*), and somewhat more slowly in the ovariectomized animals ig. 2, *B*). It is apparent that the early increase in liver mass (from to 16 hours) was almost entirely due to fatty infiltration.

During the period of the most rapid increase in liver mass (20 to 30 ours), deposition of protein and water proceeded at an accelerated rate

(Figs. 2, *A* and 2, *B*). Histological examination of sections of liver tained from animals at this period of time after partial hepatectom vealed that this rapid rate of protein and water influx was paralleled b inception of mitotic activity in the hepatic cells. It may also be that lipide accumulation continued rapidly at the same time. In groups of rats, the rate of increase of protein and water in the liver

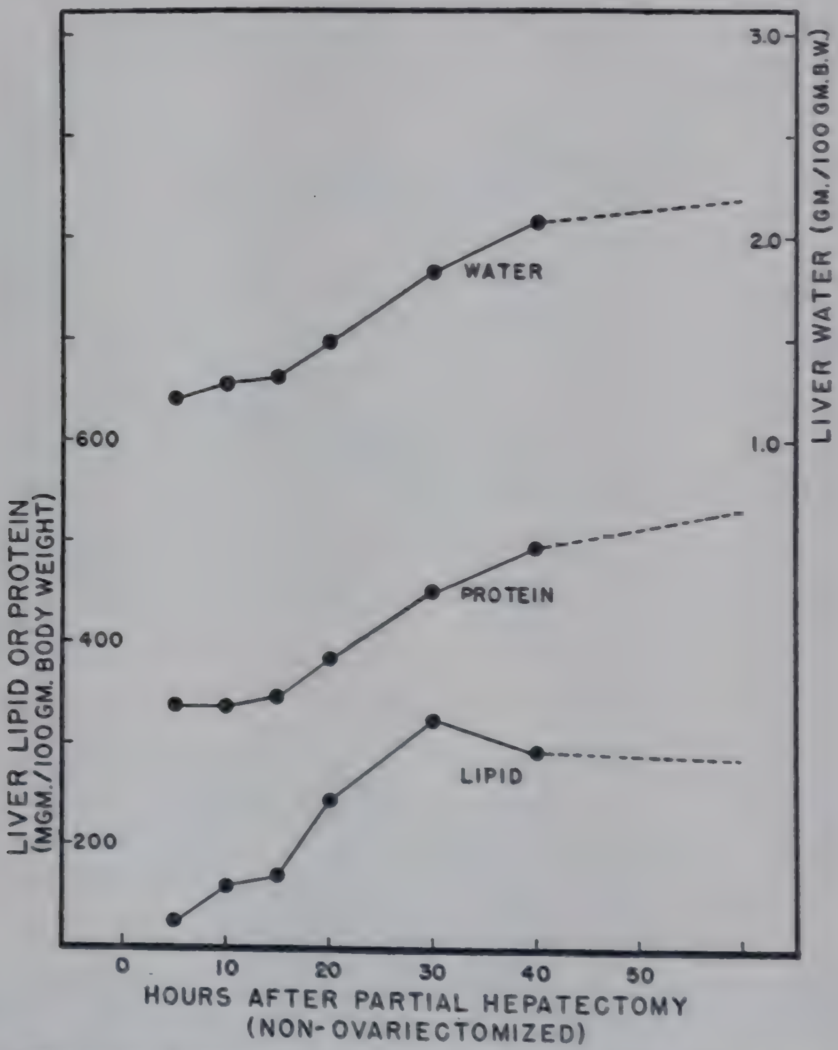

FIG. 2, *A*. The rate of restoration of liver protein, water, and lipide in non iectomized rats after partial hepatectomy. In this series, preoperative lev these constituents were protein 900 mg., water 3.15 gm., and lipide 267 mg. p gm. of body weight.

only slightly reduced after the first 48 hours. The reduction in the of increase of liver mass which occurred at this time (Fig. 1) was appar due to the leveling off of lipide deposition at approximately the n value of liver lipide concentration (*i.e.*, 250 to 300 mg. per 100 g body weight).

The concentrations of liver constituents at varying times after p atectomy, when calculated on a percentage basis, were apparently kedly influenced by previous removal of the ovaries. The prompt bilization of lipide to the liver stump remaining after partial hepatec- y in the non-ovariectomized group is shown in Fig. 3. In the ovariec- ized animals, however, there was an initial lag in lipide deposition, ch was followed later by a rate of lipide accumulation in the liver

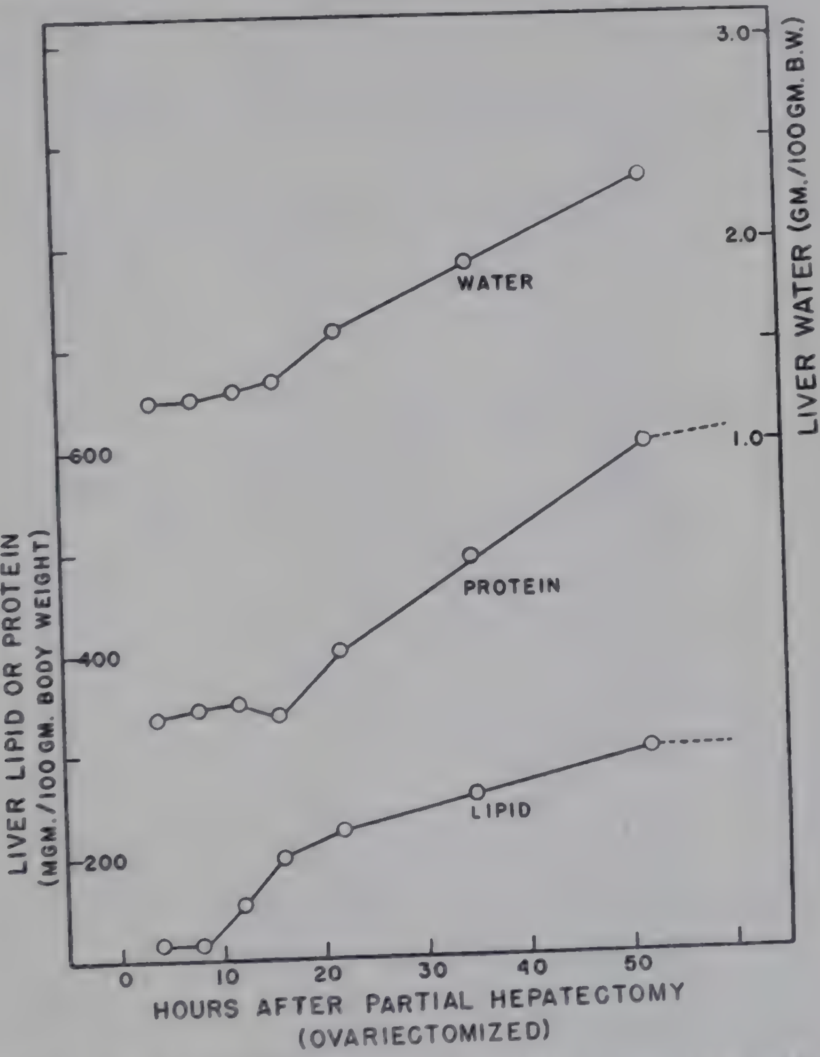

FIG. 2, *B*. The rate of restoration of liver protein, water, and lipide in ovariecto- ized rats after partial hepatectomy. In this series, preoperative levels of these onstituents were protein 888 mg., water 3.09 gm., and lipide 279 mg. per 100 gm. of ody weight.

imilar to that observed in the control group. This resulted in the failure f the ovariectomized rats to attain the high liver lipide levels seen about 0 hours after liver removal in the non-ovariectomized group. The dif- erences in liver lipide concentration in the two groups were statistically ignificant at the following time intervals after partial hepatectomy: to 10 hours, 20 to 22 hours, and 30 to 52 hours (*cf.* Table I). It will be noted in Table I that differences in the various liver constituents in two groups were, in general, of greater statistical significance when culated on a per cent basis than when related to the body weight. apparent discrepancy was probably due to the fact that body weig after partial hepatectomy were quite variable as a result of individ differences in food intake, spontaneous activity, etc.

The changes in the per cent of protein and water in the liver during early period of restoration are shown in Figs. 4 and 5. Apparent ferences between the two groups of animals were seen throughout period. Thus, in the control group, there was a depression in the conc

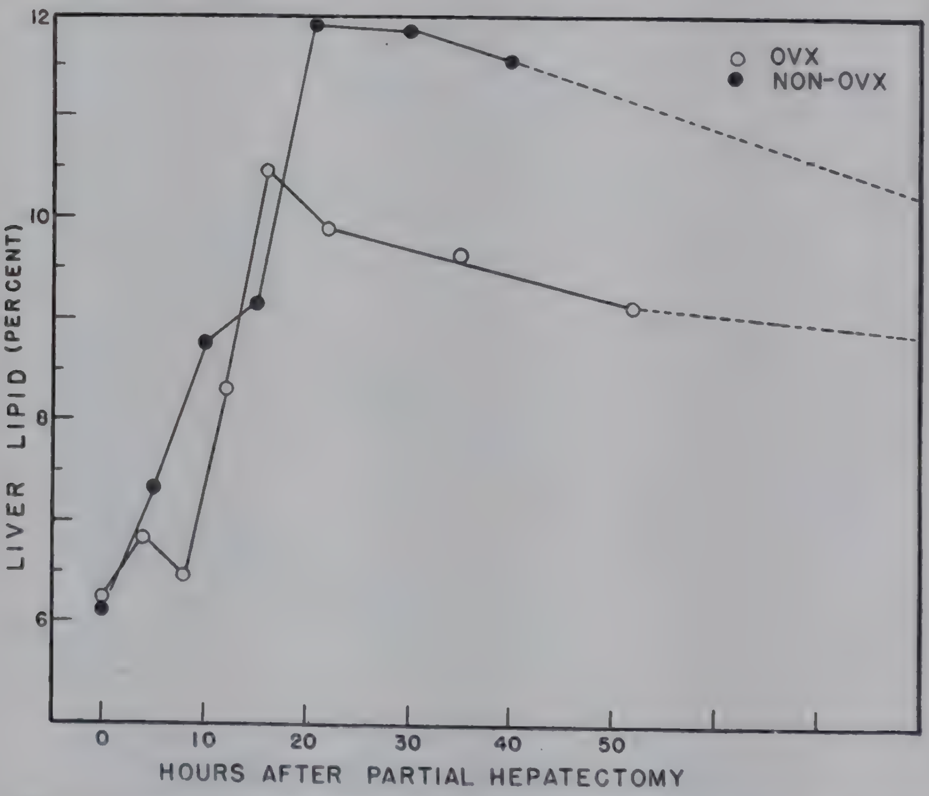

Fig. 3. The influence of ovariectomy on lipide concentration in the regenerati liver after partial hepatectomy.

tration of protein and water concomitant with the immediate mobiliz tion of lipide (Fig. 3). In the ovariectomized group, on the other hand transitory rise in protein and water levels occurred during the first 8 10 hours. Shortly thereafter, both groups of animals exhibited reductio in the relative amounts of liver protein and water present; these lev reached a minimum at a time when the per cent lipide levels were ma mum. Subsequently, protein, water, and lipide concentrations all beg to return to normal. Thus, while previous ovariectomy caused a delay accumulation of lipide in the livers of partially hepatectomized rats (Fi 2, *A*, 2, *B*, and 3), the rate of protein and water deposition in this gro

TABLE I

*Influence of Ovariectomy on Chemical Composition of Regenerating Liver**

Time interval, hrs. after partial hepatectomy	Lipide		Protein		Water	
	per cent	*mg. per 100 gm. body weight*	*per cent*	*mg. per 100 gm. body weight*	*per cent*	*gm. per 100 gm. body weight*
0	−0.13 ± 0.20 (14)	−12.0 ± 12.1 (14)	0.38 ± 0.36 (44)	12.5 ± 19.1 (44)	0.6 ± 0.3 (45)	0.06 ± 0.10 (37)
4–5	0.51 ± 0.42 (11)	10.8 ± 5.7 (11)	−0.56 ± 1.18 (11)	0 (11)	−1.0 ± 0.7 (8)	−0.08 ± 0.22 (8)
8–10	2.29 ± 0.76† (9)	47.9 ± 15.5† (9)	−1.25 ± 0.61 (9)	−6.3 ± 18.8 (9)	−1.7 ± 0.7‡ (9)	−0.04 ± 0.06 (9)
15–16	−1.32 ± 1.17 (12)	−29.0 ± 17.3 (12)	1.63 ± 0.73 (13)	6.3 ± 13.0 (13)	−0.1 ± 1.0 (9)	−0.01 ± 0.11 (9)
20–22	2.30 ± 0.79† (10)	31.9 ± 17.8 (10)	0.06 ± 0.38 (10)	−18.8 ± 18.8 (10)	−1.0 ± 0.8 (8)	−0.10 ± 0.06 (8)
30–52	2.29 ± 0.75† (19)	53.5 ± 20.7‡ (19)	−1.81 ± 0.34† (22)	−62.5 ± 25.0‡ (22)	−2.5 ± 0.8† (21)	−0.14 ± 0.11 (21)

* The figures shown are the arithmetical differences of the means (between non-ovariectomized and ovariectomized animals) and the standard errors of the differences. A positive value indicates that the figure for the non-ovariectomized group exceeded that for the ovariectomized animals; a negative value indicates the converse. The figures in parentheses represent the total number of observations in the groups being compared.

† The P value of the difference is <0.01.

‡ The P value of the difference lies between 0.01 and 0.05.

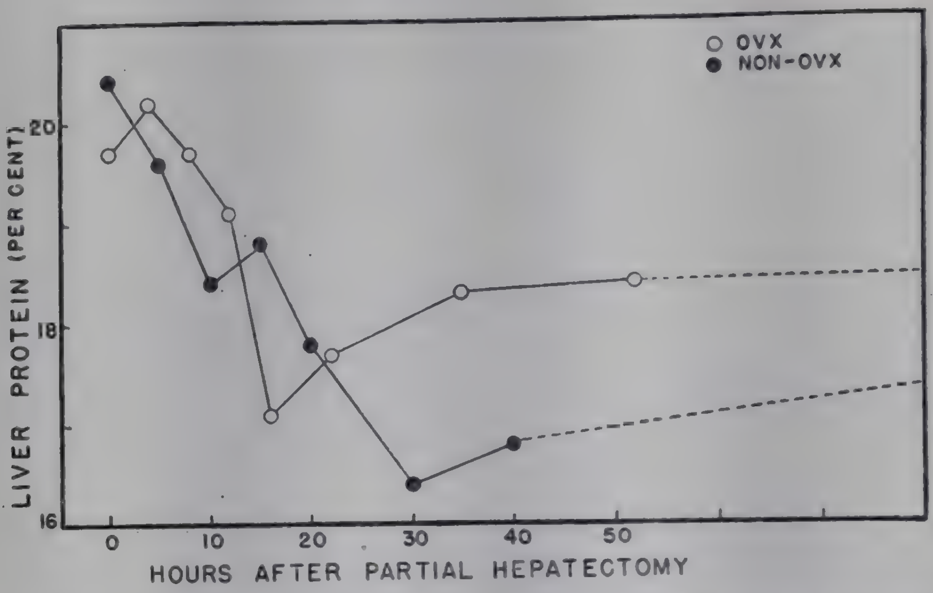

FIG. 4. The influence of ovariectomy on protein concentration in the regenerat liver after partial hepatectomy.

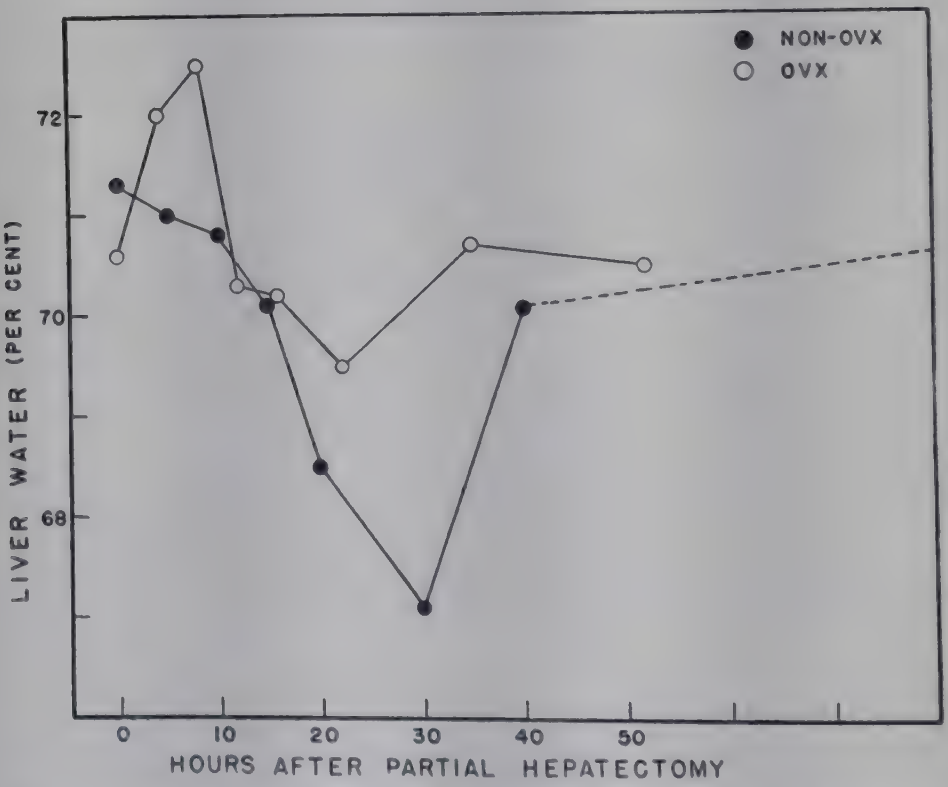

FIG. 5. The influence of ovariectomy on water concentration in the regenerat liver after partial hepatectomy.

surpassed that in the controls (Figs. 4 and 5). Table I reveals that th differences were statistically most significant 30 to 52 hours after par hepatectomy.

DISCUSSION

The classical study of Higgins and Anderson (1) revealed that restoration of hepatic tissue after partial hepatectomy in the rat was a reproducible process which might be employed in the study of growth. These workers first demonstrated the initial fall in liver water after this operation. Brues, Drury, and Brues (3) observed the progressive decline in liver nitrogen which reached a minimum at the end of the 2nd postoperative day. Lipide infiltration into the liver stump was inferred, but not demonstrated. Direct measurements of the lipide distribution in rat liver after partial hepatectomy have been made by Ludewig, Minor, and Hortenstine (19), who observed a marked influx of fat concomitant with a fall in the phospholipide-neutral fat ratio. Bogetti and Mazzocco (4) and Berman *et al.* (20) found similar early increases in total lipide. The present data further substantiate the observation that the composition of the early increase in liver mass after partial hepatectomy is mainly lipide. It may be concluded that the initial restoration of the liver is primarily an increase in mass due to lipide deposition, and, only thereafter, a readjustment to normal composition.

Mitotic figures, although extremely rare in normal liver (21), are conspicuous in livers regenerating after various types of injury, including damage by toxins, chemical agents, etc. (*cf.* Fishback (22), Eschenbrenner and Miller (23)). In the present study, the inception of mitotic activity in the hepatic cells was well correlated with the initiation of protein deposition. Both processes began about 24 hours after partial hepatectomy, and after a short period of rapid acceleration, leveled off to more moderate rates. These observations are similar to those of Brues, Drury, and Brues (3), Brues and Marble (21), and Gurd, Vars, and Ravdin (13). Christensen, Rothwell, Sears, and Streicher (24) have recently observed an early increase and later decline in liver amino acid concentration after partial hepatectomy. They also noted a close association of the period of ncrease in amino acid concentration with the time of the most active iver growth.

The relatively prompt accumulation of liver lipide is the primary reaction to many types of liver injury, including partial hepatectomy, and is ollowed shortly by mitotic activity and protein deposition. These events re striking in their time sequence. It has been suggested frequently that ccumulation of liver fat *per se* may impair hepatic function, possibly by rowding out the active cytoplasm in the liver cell (*cf.* (25, 26)). This oncept is not substantiated by the work of Wells (27) and of Ennor (28) n which very fatty livers caused by toxic damage exhibited no impairnent of metabolic activity *in vivo* or *in vitro*. The results of the present investigation support the suggestion that infiltration of liver lipide following hepatic injury may be an integral part of the restoration phenomeno The initial lipide accumulation, which accounts for virtually all of th increase in liver mass during the first 24 hours following partial hepate tomy, could furnish the energy for subsequent growth and readjustment the liver to normal composition. A similar mobilization of lipide to th liver accompanies the nitrogen retention seen following the injection adenohypophyseal extracts possessing growth-promoting activity (c (29)).

The relationship between the rate of lipide infiltration and true growt of the liver stump after partial hepatectomy does not appear to be strict quantitative in nature. Thus, notwithstanding the diminished rate fat accumulation in the ovariectomized animals, the rate of protein ar water deposition was greater in this group than in the controls. In add tion, the restoration of liver tissue of normal composition was approache earlier in the animals deprived of ovarian secretions. These observ tions do not provide evidence against the suggestion that fat may supp energy for growth, for, even in the ovariectomized group, accumulation liver lipide preceded sustained protein and water deposition by at least hours.

It has been suggested that the estrogens may have a fat-mobilizing e fect under certain conditions. György and his associates (30) have show that administered estrogen decreased the fatty infiltration of the liver rats maintained on a diet low in lipotropic factors. Lorenz, Entenma and Chaikoff have demonstrated accelerated fat metabolism in certa avian species during periods of increased ovarian activity; this was chara terized by lipemia (31) associated with increased liver lipide levels (3 The increase in blood lipides could also be provoked by the administrati of estrogen to male and immature female birds (33). In the present stud the presence of ovarian secretions apparently enhanced liver lipide depo tion in the rat immediately after partial hepatectomy. These results a in agreement with the observations of MacKay and Carne (34) that mo fat was deposited in the livers of female rats than in those of male ra during the first 24 hours after partial removal of the liver. The observ tions of Deuel and coworkers (35) on the inherent sex differences in metabolism lend additional support to the concept that ovarian secretio may play a direct rôle in promoting lipide mobilization.

SUMMARY

The influence of ovariectomy on the composition of liver during ea hepatic regeneration has been studied following partial hepatectomy the rat.

An apparent lag in the restoration of liver mass for the first 4 to 8 hours ollowing operation was noted in the ovariectomized group. This deiciency was due to failure of prompt mobilization of lipide to the liver in hese animals. Lipide accumulation in both groups was responsible for irtually all of the early increase in liver mass (up to 24 hours postoperaively). Due to the initial lag in lipide mobilization in the ovariectomized group, liver lipide failed to reach as high maximum levels as were observed n the control series. The data suggest that ovarian secretions promote ipide mobilization to the regenerating liver.

Protein and water deposition was closely correlated within each group, and was demonstrable by about 24 hours after operation, paralleled by the beginning of mitotic activity. The restoration of liver protein and water occurred at a significantly greater rate in the animals deprived of ovarian secretions.

The rate of increase in liver mass approached plateau levels in both groups of animals at about 48 hours, in spite of a continuing increase in protein and water deposition. This was due to the leveling off of liver lipide accumulation at approximately the normal value (250 to 300 mg. per 100 gm. of body weight). Readjustment to normal liver composition was thus approached.

The data support the suggestion that the relatively prompt accumulation of liver lipide as the primary reaction to most types of hepatic injury, including partial hepatectomy, may be an essential part of the restoration process, and may provide energy for growth and repair.

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PHOTOMETRIC METHOD FOR THE DETERMINATION OF INULIN IN PLASMA AND URINE*

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t is generally accepted that, of the substances used to test glomerular ation, inulin is the most satisfactory. For the determination of inulin rance, inulin is injected intravenously and the concentration of inulin blood plasma and urine is estimated. This is accomplished by hods based upon the reducing power of the fructose derived from the lrolysis of inulin (1), or upon the reaction of an inulin hydrolysate with henylamine, giving a blue color (2, 3) or with resorcinol, giving a red (4, or yellow (6) color.

he methods for the determination of inulin by reduction and by the nation of a blue color with diphenylamine have the serious limitation t glucose is an interfering substance which must be removed by treatnt with yeast. These procedures are long and cumbersome because of necessity of preparing a starch-free yeast, treating the solution conning the inulin with yeast, and running a hematocrit on the yeast susnsion to determine the degree of dilution of the plasma or urine by the st.

Kruhøffer (6) has reported that the results with the diphenylamine thod are not as accurate, or as reproducible, as those based upon the ction of fructose with alcoholic resorcinol in the presence of hydrooric acid. The latter reaction, as shown by Roe (7), is specific for fruce in the presence of the amounts of glucose found in normal blood and ne, and it has been used as the basis of several methods for determining lin in plasma and urine. Of the latter methods the first published was t of Steinitz (4) who applied Roe's method for the determination of ctose in blood and urine to the measurement of inulin. This author nd that maximum absorption of the color used is at 510 mμ and that Beer-Lambert law was applicable only in narrow ranges of inulin contration. He used several standard inulin solutions in order to compe the unknown with the standard that most closely matched it. This k was followed by that of Hubbard and Loomis (5) who varied the cedure by using half the volumes employed by Steinitz and reading

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the color at 450 mμ instead of at 510 mμ. Kruhøffer (6) has mod Roe's method by combining the resorcinol and hydrochloric acid tions in one reagent and, instead of heating at 80° for 8 minutes, he the solutions for 1 hour to obtain a yellow color which is read at 450 To Kruhøffer's method the same objection can be raised as that menti above; namely, that it is necessary either to remove the glucose with y or to determine the concentration of glucose present and correct for amount of inulin which it represents. Kruhøffer's method also call the unwieldy procedure of boiling the inulin-reagent mixture for 1 ho sealed tubes.

With the prevailing techniques for the determination of inulin at o disposal, a new method for carrying out this procedure would not be j fied unless there were distinct advantages inherent in it. Our reason presenting the proposed method are as follows: It is a rapid, simpl procedure which does away with treatment with yeast and is highly curate. The color development follows the Beer-Lambert law thro out the working range of inulin concentrations used in studies of kid clearance The reagent used by Roe (7) has been changed by dissol the resorcinol in acetic acid containing thiourea, instead of in alcoh modification that appreciably increases the depth of color. The mod reagent is also a better mixture for the hydrolysis of inulin, as it cont glacial acetic acid in place of ethyl alcohol.

Technique of New Method

Reagents—

1. Resorcinol-thiourea reagent. Dissolve 0.1 gm. of resorcinol and gm. of thiourea in 100 cc. of glacial acetic acid. This reagent should kept in a brown bottle.

2. 30.0 per cent hydrochloric acid. To 1 part of distilled water ad parts of concentrated HCl, sp. gr. 1.19.

3. Standard inulin solution. Prepare a stock inulin solution contai 1 mg. per cc. Dilute this 1:50 to obtain a working inulin standard taining 0.02 mg. per cc.

4. Somogyi deproteinizing reagents (8). Solution I, 10 per solution of $ZnSO_4 \cdot 7H_2O$; Solution II, 0.5 N NaOH. These solutions m neutralize each other precisely, volume for volume, when titration is formed with phenolphthalein as indicator.

Procedure

For Plasma—To 1 part of heparinized plasma add 15 parts of disti water, 2 parts of 10 per cent $ZnSO_4 \cdot 7H_2O$, and 2 parts of 0.5 N Na Filter through a filter paper free of inuloid material. Place 2 cc. of ate in a colorimeter tube; also pipette into a similar tube 2 cc. of the ıdard solution containing 0.02 mg. of inulin per cc. Add 1 cc. of the rcinol-thiourea reagent and 7 cc. of the 30 per cent hydrochloric acid ach tube. The solution is mixed well by gentle shaking and the tubes placed in a water bath at 80° for exactly 10 minutes. Remove the es and bring to room temperature by immersing in tap water for roximately 5 minutes; during this time it is best to keep the tubes in dark. Dry the outside of the tubes and read in a photoelectric colorim- , using a filter with a maximum transmission of light at 520 The colorimeter is set by the use of a reagent blank prepared as cted below.

or Urine—The urine is treated with the Somogyi deproteinizing rea- ts as above under the procedure for plasma; this removes proteins, if sent, and at the same time decolorizes the urine. The filtrate is then ted with distilled water until it contains between 0.25 and 3.0 mg. per t of inulin. Diluted urine filtrate is treated as outlined above plasma filtrate.

or Reagent Blank—The photoelectric colorimeter is set at 100, or the point, by the use of a reagent blank which is prepared by substituting . of distilled water for the 2 cc. of filtrate and proceeding according to directions given for plasma.

or Plasma and Urine Control Blanks—The plasma blank and urine nk are prepared by making filtrates of the control plasma and urine ained before the administration of inulin. These blank values are ectly determined by treating the filtrates in the manner described under procedure for plasma; this is usually accurate enough for routine clini- work.

or more accurate work an additive blank can be used for the control sma and urine. The additive blank is prepared by adding a known ntity of inulin to the control plasma and urine filtrates in order to bring developing color within range of greater accuracy of the method. An al quantity of inulin is simultaneously added to a similar volume of er to serve as the reference standard for the control filtrates. Then h the control filtrates with the inulin added and the reference standard treated with the resorcinol-thiourea solution and hydrochloric acid as en under the directions for plasma. Generally the plasma blank is ger than the urine blank; the latter is usually so minute that in most es it can be disregarded.

alculations—The calculations are the same as those for any determina- n in a photoelectric colorimeter.

$$\frac{\text{nsity of unknown}}{\text{nsity of standard}} \times \text{mg. standard} \times \frac{100}{\text{cc. plasma or urine}} = \text{mg. inuloid material per 100 cc. plasma or urine}$$

From this value subtract the respective blank values for plasma and u
to arrive at the correct concentration of inulin in both fluids.

Mg. % total inuloid material in plasma or urine *minus* mg. % inuloid material in plasma blank or urine blank = mg. inulin per 10 plasma or urine

If an additive blank for plasma and urine is used, the calculations
volve subtracting the value of the reference standard from the valu
the additive blank to ascertain the plasma or urine control value.

DISCUSSION

With this method, it is necessary to measure all reagents accurately,
the depth of color obtained is dependent on the amounts of resorci
thiourea, glacial acetic acid, and HCl present, as well as on the concent
tion of inulin. All tubes should be placed in the 80° water bath and
moved to cold water exactly at the same time. The red color produ
is dependent upon the velocity of the reaction at an elevated te
perature; therefore, identical heating and cooling conditions should ex
in both the standard and unknown solutions. After cooling to room te
perature, the tubes should be read in the photoelectric colorimeter wi
out delay, as the color begins to fade after standing for 30 minutes.

The red color obtained with this method is of an excellent quality
photometric work. As can be seen in Fig. 1, the curve obtained by t
new method is in good agreement with the Beer-Lambert law, and t
red color formed by the present method is more intense than the co
produced by Roe's original reagent for fructose (7). The curve in Fig.
prepared by Roe's original method shows poor agreement with the Be
Lambert law, an observation in accord with the findings of Steinitz (
The excellent result obtained with the present method is apparently d
to the use of acetic acid instead of ethyl alcohol in the reagent used for h
drolysis and color production. The increase in the intensity of the co
and the close adherence to the Beer-Lambert law make the propos
method more sensitive and accurate than previous procedures (4, 5) bas
upon the same principle.

An absorption curve of the color produced with this method is shown
Fig. 2. Maximum absorption occurs in the range of 510 to 520 mμ.

Experiments upon the recovery of inulin added to the plasma and ur
of fasted patients are presented in Table I. These results show that t
method is highly reliable for the determination of inulin in plasma a
urine. In addition they show that the Somogyi deproteinizing reage
do not remove a determinable amount of inulin from urine or plas
during the preparation of the filtrate. With this procedure the detern
nation of very small amounts of inulin can be accurately carried out; a

sma concentrations as high as 60 mg. per cent are within the range of method without further dilution than that involved in deproteiniza- n.

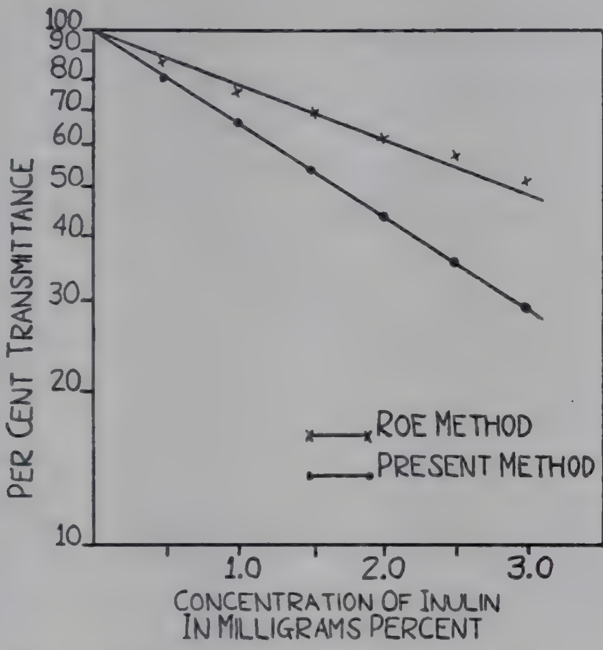

Fig. 1. Curves showing agreement with the Beer-Lambert law

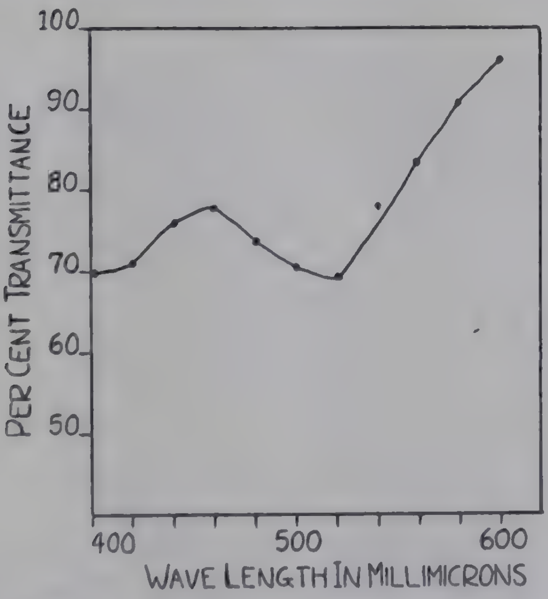

Fig. 2. Absorption curve of the chromogen formed by a 2 mg. per cent fructose ution, Beckman spectrophotometer.

There is no error from normal concentrations of glucose in this ocedure because correction is made for the chromogenic value of glu- se by determining the inuloid material in the control blank, which may expected to remain fairly constant during the experimental test on a fasting patient. However, in the determination of a glucose T_m (tub secretory mass) or in a diabetic patient with a high blood sugar, the of a control blank would not give an accurate result. To show the lim tions of this method in the presence of abnormal concentrations of gluc the data of Table II are presented. The chromogenic value of gluc under the conditions of this method is small. The effect of glucose is diminished by the dilution required in preparing filtrates. As show Table II, the additive error with a blood sample containing 1000 mg glucose per 100 cc., if a dilution of 1:20 is used in preparing the filtr

Table I

Recovery of Inulin Added to Plasma and Urine

Plasma			Urine*		
Added	Recovered	Per cent recovery	Added	Recovered	Per cent recovery
mg. per cent	*mg. per cent*		*gm. per cent*	*gm. per cent*	
25.00	25.50	102	2.50	2.52	101
12.50	12.30	99	1.25	1.27	102
6.25	6.18	99	0.625	0.596	96

* The urine was diluted 1:100, including the Somogyi deproteinization step.

Table II

Data Showing Additive Error When Inulin Is Determined in Presence of Diffe Concentrations of Glucose by Proposed Method

Glucose concentration	Chromogenic equivalent as inulin	Per cent error with 12 mg. per c inulin solution
mg. per 100 cc.	*mg. per 100 cc.*	
100	0.00–0.05	0.00–0.42
200	0.10–0.15	0.83–1.25
250	0.15–0.23	1.25–1.91
500	0.27	2.25
1000	0.55	4.58

and the calculations are based on the desirable 12 mg. per cent concent tion of plasma inulin, would be 4.58 per cent. These data show that error due to glucose is not appreciable until fairly high concentrations glucose in blood and urine are encountered.

SUMMARY

A photometric method for the determination of inulin in plasma urine has been developed. The method is based upon the red color p duced by warming an inulin-containing filtrate mixed with HCl an

gent containing resorcinol and thiourea in glacial acetic acid at 80° for minutes.

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THE INFLUENCE OF PTEROYLGLUTAMIC ACID ON GLYCINE AND ON PORPHYRIN METABOLISM*

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In earlier communications we have presented evidence which we con-
ered to be suggestive of a relationship between pteroylglutamic acid
GA) or the antipernicious anemia principle and porphyrin metabolism
2). Some of the evidence has been indirect and not entirely convincing.
owever, the data in this communication offer unequivocal support to our
pothesis, and further provide insight into the mechanism of action of
A in influencing the production of porphyrins. In a previous paper (3)
was implied that PGA may also influence the destruction of such por-
yrin-containing compounds as hemoglobin, through its inhibitory action
certain flavin-containing enzymes.

It has been shown by Shemin and Rittenberg (4) that portions of the
cine molecule are incorporated into the protoporphyrin of hemoglobin.
nce rats excrete a considerable amount of protoporphyrin, which is
ought to be produced in excess of their requirement for hemoglobin
oduction (5), they are useful subjects for the study of porphyrin me-
bolism. In the present experiments the effect of PGA on porphyrin
cretion has been studied with animals on a standard purified diet and
a purified diet containing sodium benzoate.

Griffith (6) established that a reduction in the growth rate of rats follows
e inclusion of sodium benzoate in their diets. This reduction was readily
ercome by the addition to the diet of sufficient glycine to detoxify the
nzoate. He also found that a partial restoration of growth on the
nzoate-containing diets could be brought about by allowing the animals
have access to their feces or by increasing the amount of yeast used as a
urce of the vitamin B complex. In the present experiments the inclu-
on of PGA in the diets of sodium benzoate-fed rats was found to result

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es of Health, United States Public Health Service, and from the Williams-Water-
n Fund of the Research Corporation. The pteroylglutamic acid used was sup-
ed by the Lederle Laboratories Division, American Cyanamid Company.

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alysis of the data, of Doris Warren in the microbiological assays, and of William
Martindale in the care of the animals.

in a similar stimulation of growth. PGA may affect the productio porphyrins in the animal body through this effect on glycine metaboli

Since glycine may be important as a precursor of uric acid (7), doub one function of PGA in relation to purines may also be mediated thr its influence on this amino acid.

EXPERIMENTAL

The experimental animals were litter mate rats of the Sprague-Da strain, weighing initially 41 to 74 gm. They were housed in indivi cages on a raised screen which allowed the feces to fall through for co tion. Food and water were available at all times. During certain per as indicated below the animals were transferred to metabolism cage the purpose of collecting urine. The basal diet consisted of Labco ca 18 gm., sucrose 75 gm., hydrogenated vegetable oil 3 gm., cod liver gm., and salt mixture (8) 2 gm. To each kilo were added inositol 1 choline chloride 1 gm., thiamine chloride 5 mg., riboflavin 5 mg., pyrido 5 mg., calcium pantothenate 10 mg., nicotinic acid 20 mg., menad 0.25 mg., and biotin 0.024 mg. 3 per cent sodium benzoate replace equivalent weight of sucrose in three of the diets employed (Groups IV, and V) and 2.0 per cent glycine replaced an equivalent weigh sucrose in the diet of Group V. The diets of Groups II and IV conta 5 mg. of PGA per kilo.

Food consumption and body weights were recorded at 4 day inter and fecal collections were made at the same time. The pellets from group were combined and dried overnight in an oven at 100°. The d feces were then weighed, ground in a mortar, and a sample weighed a rately for porphyrin determination.

In the early part of the experiment several 4 day collections were accu lated before the porphyrin extractions were made. However, it was fo that this procedure gave lower values than determinations made imm ately after drying. Consequently, all of the later determinations made as soon as possible after the heating.

Porphyrin was determined on the sample by extraction with 5 per HCl. The acid was neutralized to Congo red in the presence of the material and several ether extractions carried out. The combined e layers were washed with water and the porphyrin reextracted with s portions of 5 per cent HCl until no further porphyrin could be removed f the ether. The quantity of porphyrin was calculated from the optical sity of the HCl solutions at 408 mμ determined on a Beckman spectro tometer. No effort was made to remove the small amount of coproporp rin normally present, which has a sharp absorption maximum near mμ. The results obtained agreed well with similar determinations m repeated extraction of the porphyrin from the feces by means of glacial ic acid followed by partition between ether and dilute HCl.

rinary porphyrin was extracted by a procedure similar to that of ington and Hemmings (9), except that 5 per cent HCl was used for the nd extraction instead of 0.5 per cent HCl. Determinations of the roporphyrin solutions were made at 402 mμ on the Beckman spectrotometer. The values obtained agree well with those published by Scudi Hamlin (10).

teroylglutamic acid was determined microbiologically (11) by use of *ptococcus faecalis* (American Type Culture Collection No. 8043) on 1 samples of the dried feces.

ematological studies on peripheral blood were carried out on all animals tandard procedures between the 61st and 63rd experimental days.

RESULTS AND DISCUSSION

he average growth curves of rats consuming each of the diets are shown ig. 1. The removal of sodium benzoate from the diet of Group III for a lay period was followed by an immediate resumption of rapid growth. s removal was deemed necessary in order to maintain a large enough nber of animals in the group for control purposes. The mortality on sodium benzoate control diet is usually quite high after the 3rd week, ecially on a highly purified diet, as was shown by preliminary experints. The food consumption and efficiency of food utilization in our es closely resemble similar data obtained by Griffith (6) and are not reled here.

able I gives representative data on the fecal dry weight, fecal porphyrin, fecal PGA, as well as coproporphyrin on 2 day urinary specimens coled from the indicated number of animals from each group. Data obned during the period of removal of sodium benzoate from the diet of up III were omitted from Table I. Likewise the data on fecal porphyfrom the early part of the experiment were omitted for reasons discussed ve. The relative proportions of porphyrin in the early samples, howr, were very similar to the later and more accurate determinations given able I.

lematological data obtained at the end of the experimental period are sented in Table II. Statistically significant differences were observed he average white blood cell counts as indicated in Table II.

he addition of sodium benzoate to the purified diet led to a lowered l porphyrin output which persisted throughout the experiment. The uction was manifest at the end of the first 4 day period of collection, ce it cannot be due solely to the lower weight of the sodium benzoate-fed controls. These data are in accord with the known function of glycin protoporphyrin production (4, 12).

The reduction in porphyrin production and in the growth of the sodi benzoate-fed animals was largely overcome by the inclusion of 500 γ PGA per 100 gm. of ration (Fig. 1 and Table I). The addition of 2.0 cent glycine in the absence of added PGA completely overcame the red

TABLE I

Influence of 3 Per Cent Sodium Benzoate and of Pteroylglutamic Acid (PGA) o Fecal Weight, Fecal and Urinary Porphyrin, and Fecal Pteroylglutamic Acid Excretion in Rats

Group No.	Diet	No. of animals	Averages							
			Fecal dry weight per rat per day		Fecal PGA*		Fecal porphyrin, 37–64th day		Urinary po phyrin for 2 period†	
			1–20th day	37–64th day	Per gm. dried feces	Per rat per day	Per rat per day	Per gm. food intake	Total	Per gm.
			gm.	gm.	γ	γ	γ	γ	γ	γ
I	Basal (control)	12	0.138	0.250	4.0	0.78	41.8	2.4	4.2	2
II	“ + 500 γ PGA per 100 gm.	12	0.190	0.221	8.6	1.44	50.0	3.2	5.6	3
III	Basal with sodium benzoate	13	0.100	0.179	2.3	0.35	27.0	2.9	3.0	2
IV	Basal with sodium benzoate + 500 γ PGA per 100 gm.	12	0.094	0.194	8.1	1.57	37.2	3.2	4.6	2
V	Basal with sodium benzoate and 2.0% glycine	12	0.173	0.234	3.0	0.69	52.2	4.2	4.0	2

* Average of determinations on collections from four of the 4 day periods.

† Collections were made between the 37th and 48th experimental day. The nu ber of animals from which collections were obtained and pooled were, respective Group I, nine; Group II, nine; Group III, twelve; Group IV, twelve; Group twelve.

tion in fecal porphyrin production but sufficed to restore fully the grow of the animals only during the first 40 days of the experiment, as sho in Fig. 1. Thereafter the growth rate of this group declined apprecia below that of the controls not receiving sodium benzoate.

The addition of PGA to the basal diet increased significantly the fecal p phyrin throughout the experimental period, although no effect on grow was observed.

The changes observed in the urinary coproporphyrin (Table I) were nilar to those in fecal porphyrin except that the extra glycine in the diet Group V did not appear to enhance the coproporphyrin output of this oup appreciably over that of the control group. The coproporphyrin ta, however, are too meager to establish this conclusion.

The growth data presented here resemble those obtained by Daft (13) for her amino acid deficiencies and for low protein diets. This suggests that e effect is a general one on protein metabolism and may be similar for iny dietary or metabolically produced amino acids. The additional idies conducted on the present series of experimental animals enable one draw some conclusions as to the probable mechanism of action of PGA producing its sparing effect.

TABLE II

Hematological Data on Peripheral Blood of Rats Fed Purified Diets with and without Sodium Benzoate and Pteroylglutamic Acid

roup No.	Diet	No. of animals	Average			
			Erythrocytes	Leucocytes	Hemoglobin	Reticulocytes
			millions per μl.	*thousands per μl.*	*gm. per 100 ml.*	*per cent of erythrocytes*
I	Basal (control)	10	8.5	17.2*	14.2	4.1
II	" + 500 γ PGA per 100 gm.	10	8.4	19.7	15.2	3.0
III	" with 3% sodium benzoate	12	7.9	14.4	14.7	5.0
IV	Basal with 3% sodium benzoate + 500 γ PGA per 100 gm.	11	8.8	17.2	14.6	3.1
V	Basal with 3% sodium benzoate + 2% glycine	10	8.4	14.8	14.2	2.8

* A difference of 2.7 × 10^3 white blood cells per microliter may be considered to be statistically significant (P = 0.01) difference from the average of the controls roup I).

Griffith (14) has discussed the possible mechanisms of the growth stimution afforded by coprophagy in his experiments. He concluded that the tra vitamin B complex obtained by the animals in this way was entirely sponsible. The data given in Table I provide evidence bearing on the ssibility that additional glycine could be obtained by intestinal synthesis. he fecal dry weights of the control animals consuming a ration with sucrose the source of carbohydrates are about one-fourth of the fecal weights obrved in the similar experiments of Orten and Keller (5), who employed a et containing dextrin. It will be noted that sodium benzoate brought out a further decrease in fecal dry weight which was not affected by the dition of PGA to the diet. Since the feces from rats consuming a purified

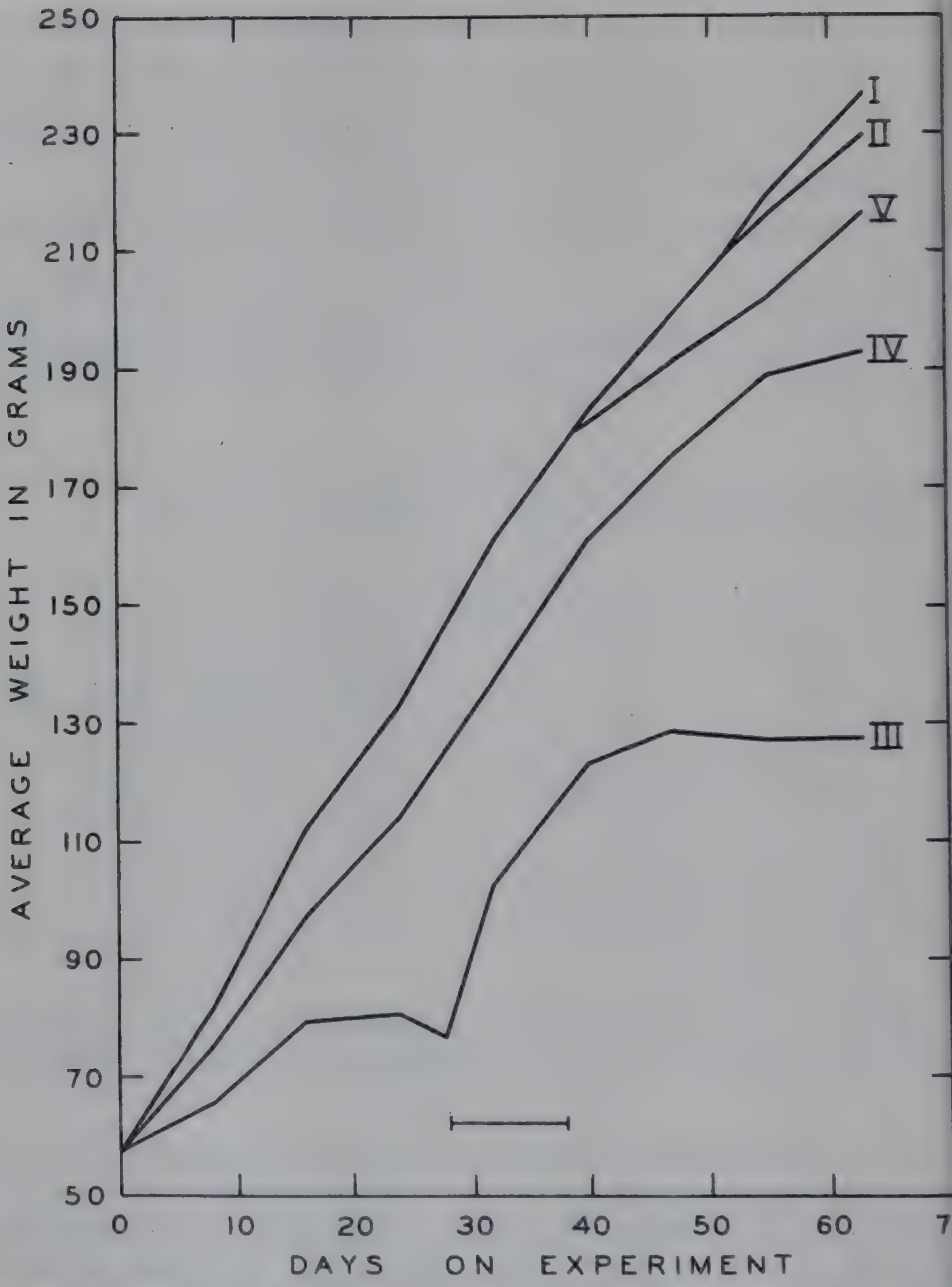

FIG. 1. Growth curves of rats receiving a standard purified diet. Curve I, w
out pteroylglutamic acid (PGA); Curve II, with 5.0 mg. of PGA per kilo of
Curve III, with 3 per cent sodium benzoate; Curve IV, with 3 per cent sodium
zoate and 5.0 mg. of PGA per kilo of diet; and Curve V, with 3 per cent sodium
zoate and 2 per cent glycine. The numbers of animals were respectively tw
twelve, thirteen, twelve, and twelve. During the period of time represented b
horizontal bar sodium benzoate was omitted from the diet of the animals re
sented by Curve III.

diet are in large part dead bacteria, the fecal weight provides some ind
tion of the activity of the intestinal flora. It seems unlikely that

ıld stimulate bacterial production of much glycine without influencing e fecal weight. Even though excess glycine appears to restore the intes-al flora, as indicated by the fecal weights of Group V, this group does t appear to have a consistently high intestinal synthesis of PGA, as o shown in Table I. This is borne out by the reduced white blood cell ınt for this group as compared with those receiving PGA (Table II). is, therefore, improbable that glycine exercised its growth effect rough influence on the bacterial production of PGA.

It is possible that in these experiments PGA acts by reducing the rate of idative disposal of glycine. If the action were merely a stimulation of the toxication mechanism, additional glycine for the synthesis of porphyrin uld not have been made available. As suggested in an earlier communi-tion (3), this reduction in oxidation may be brought about by interference the action of glycine oxidase, a flavin-containing enzyme known to occur rat liver (15). Alternatively, the metabolic synthesis of glycine by the may have been stimulated by the PGA. Other possible mechanisms also being investigated.

Several clinical observations lend support to the view that PGA or the tipernicious anemia factor may act by reducing the activity of flavin-ntaining enzymes. Among these may be mentioned the reports of David-n and Girdwood (16) who observed the appearance or exacerbation of oflavin deficiency signs after large doses of PGA to pernicious anemia tients. West has shown that nitrogen equilibrium in humans with per-cious anemia may be maintained on previously inadequate diets after ccessful treatment with the antipernicious anemia principle (17). This uld probably be brought about by reduction of an elevated amino acid idase activity, although doubtless other enzymes are also involved.

SUMMARY

Pteroylglutamic acid (PGA) was found to increase significantly the fecal ıd probably the urinary porphyrin excretion of rats fed a purified diet.

PGA largely reversed the growth inhibition and reduction in porphyrin cretion caused by feeding 3 per cent sodium benzoate to rats maintained a purified diet. Glycine at a 2 per cent level not only wholly prevented e reduction in porphyrin excretion caused by the sodium benzoate but gnificantly increased the porphyrin above that of control animals receiving ither sodium benzoate nor PGA.

Sodium benzoate fed at a 3 per cent level reduced by about one-fourth the cal dry weight of rats fed a purified diet with or without added PGA. The duction in fecal weight was prevented by addition of 2 per cent glycine to e diet.

Rats fed 3 per cent sodium benzoate in a purified diet not containing PGA

had average white blood cell counts significantly lower than animals similar diet containing PGA. The addition of 2 per cent glycine di raise the white blood cell count of rats on a sodium benzoate-containing in the absence of dietary PGA, although it markedly increased the gr rate.

Sodium benzoate at a 3 per cent level in purified diets reduced the PGA excretion when calculated either per day or per gm. of dried fec

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THE RÔLE OF LEAD IN THE REDUCTION OF THE ERYTHROCYTE SEDIMENTATION RATE BY HYALURONIDASE

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has been recorded that the increased erythrocyte sedimentation rate R) of some pathological blood specimens, especially from patients rheumatic fever, was decreased or abolished by incubation with cer- preparations of hyaluronidase (1, 2). This effect was shown to be to changes produced on the erythrocytes and not on the plasma the erythrocytes were changed to spherocytes and so failed to form rouleaux necessary for rapid sedimentation.[1]

he action on erythrocytes was considered to be enzymatic in nature since it was dependent on time and temperature. However, the posed enzyme was not identical with hyaluronidase, since the effect the ESR did not parallel the hydrolyzing power of hyaluronidase on luronic acid.[1] The preparations of hyaluronidase, which were all le from ram or bull testes by ammonium sulfate and lead acetate tionation (3), did not all have the same activity on the ESR. It been found that these enzymes lost their activity on the ESR if they e treated with Na_2S, rather than by dialysis, for the purpose of re- ving lead (4).

he experiments reported here were performed to determine the na- of the action of hyaluronidase preparations on human erythrocytes. the hyaluronidase preparations were made and supplied by Dr. Karl yer.

Methods

amples of human blood or suspensions of washed human erythrocytes isotonic sodium chloride solution were used in all experiments and, as outine, specimens were incubated with the enzyme preparation at 37° 60 minutes. The hyaluronidase preparations were dissolved im- diately before use in the erythrocyte suspending medium. After ubation the erythrocytes were separated from the suspending medium, shed once with saline (slow centrifugation was used to avoid cell dam-

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[*] Rockefeller Foundation Fellow of the University of Sydney.

Ragan, C. A., unpublished data.

age), and suspended in twice their volume of 6 per cent low visc gelatin. The ESR in gelatin was recorded as the per cent drop in cell column after the suspension had stood for 60 minutes in Wester tubes at room temperature. Hematocrit measurements were mad large (6 ml.) tubes spun at 3000 R.P.M. for 30 minutes on a horizo centrifuge (radius, 15 cm.). Sodium and potassium determinations made on cell suspensions or suspending fluids with a flame photom employing an internal lithium standard.[2] Lead was determined by diphenyl thiocarbazone method (5), and the readings made at 510 m a Coleman model 14 spectrophotometer. Hemolysis was estimated determining the hemoglobin concentration on the suspending fluid Klett-Summerson photoelectric colorimeter.

TABLE I

Volume Changes and Potassium Loss of Erythrocytes Resulting from Their Incuba with Hyaluronidase (Preparation 34A)

48 per cent suspension of normal human erythrocytes in citrated plasma; i bated for 60 minutes at 37°; no hemolysis in any preparation.

Preparation 34A added per ml. erythrocytes	Hematocrit	Plasma electrolytes	
		K⁺	Na⁺
mg.	*per cent*	*m.eq. per l.*	*m.eq. per l.*
0	48.0	4.5	147.5
0.025	46.8	4.5	147.5
0.050	46.8	4.8	147.0
0.10	44.9	9.0	145.4
0.20	39.0	28.4	128.0

EXPERIMENTAL

Five of the ten hyaluronidase preparations tested were active when cubated with human erythrocytes, but one particularly active prep tion, known as Preparation 34A, was used in the majority of experime This preparation, in a concentration of 0.1 mg. per ml. of erythrocy had a marked effect on the ESR of either blood or erythrocyte suspensi incubated with it.

Incubation of erythrocyte suspensions with this enzyme resulted i marked diminution of hematocrit with an accompanying increase in c centration of potassium in the suspending medium. As shown in Ta I and II, the diminution in ESR, the decrease in hematocrit, and the crease in potassium concentration in the suspending medium were clo correlated. It seemed possible that the loss of potassium and water fr e cells with consequent diminution in volume and assumption of the herical form physically interfered with the formation of rouleaux. e latter are essential for the rapid sedimentation of erythrocytes.

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[2] Flame photometer built and loaned by Dr. Gilbert H. Mudge.

As a result of these observations the loss of potassium from the eryth- cytes, as reflected by the increase in concentration in the suspending edium, was selected as the most satisfactory measure of the activity of zymes (or other substances with similar action) on erythrocytes.

Since it was known that lead had been incompletely removed from some eparations of hyaluronidase (this lead was without effect on the enzy- atic hydrolysis of hyaluronic acid),[3] the concentration of this element s determined in ten preparations of hyaluronidase. Five of these ere found to contain no lead (less than 0.5 γ in 5 mg.) but the other five ontained from 0.5 to 106 γ per mg. (Table III).

TABLE II

lation of ESR to Potassium Loss from Erythrocytes Incubated with Hyaluronidase (Preparation 34A)

48 per cent suspension of washed normal human erythrocytes in isotonic sodium loride solution; incubated for 60 minutes at 37°. ESR determined in 6 per cent latin solution. Hemolysis (expressed as a per cent of the total erythrocytes in e system) was between 0.08 and 0.12 per cent in the first six tubes and 0.15 per cent the last two tubes.

Preparation 34A added per ml. of erythrocytes	Erythrocyte sedimentation rate	K^+ in supernatant after incubation
mg.	*mm. per hr.*	*m.eq. per l.*
0	63	3.7
0.02	61	3.9
0.035	56	5.1
0.06	15	16.7
0.10	1.5	26.0
0.165	0.5	31.7
0.27	<0.5	31.8
0.45	<0.5	33.7

Erythrocytes suspended in isotonic saline were incubated with these en preparations of hyaluronidase and the concentration of potassium in he supernatant fluids determined after incubation. The results of this xperiment are set out in Table III, in which it will be noted that slight emolysis occurred in several tubes, though insufficient to cause the hanges in potassium concentration. It is apparent that only those reparations of hyaluronidase containing lead were active on erythro- ytes.

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[3] Meyer, K., and Ragan, C. A., unpublished data.

It is well recognized that lead has a marked effect on erythrocyt posed to it; it is concentrated on the erythrocyte when added to blood (6) and it has a marked effect in increasing the permeabili potassium (7). Aub *et al.* (8) studied the effect of lead on the os resistance of erythrocytes to hypotonic saline solutions and reporte their resistance was greatly increased. Similar observations were on erythrocytes that had been exposed to our hyaluronidase prepar

TABLE III

Relation of Lead Content of Hyaluronidase Preparations to their Activity in C Erythrocyte Potassium Loss and Hemolysis

40 per cent suspension of washed normal human erythrocytes in isotonic s chloride solution; incubated for 60 minutes at 37°.

Preparation No.	Hyaluronidase preparation: Pb⁺⁺ content	Amount added per ml. cells: Enzyme	Amount added per ml. cells: Pb⁺⁺	K⁺ in supernatant after incubation	Hemo per c ce
	γ per mg.	mg.	γ	m.eq. per l.	
	Saline controls (4 experiments)	0	0	1.1–1.2	0.1-
57B	92	0.16	15	14.1	0.2
		0.33	30	20.4	0.3
		0.66	60	25.8	1.1
34A	106	0.05	5.3	2.0	0.1
		0.10	10.6	6.8	0.2
		0.20	21.2	17.9	0.1
81	9.5	0.50	4.8	2.0	0.1
		1.00	9.5	8.7	0.1
		2.00	19.0	23.1	0.2
38G	±0.5	10.00	±5	7.3	3.4
57C	±1.5	1.0	±1.5	1.7	0.1
61	0	10	0	0.9	0.1
43B	0	5.0	0	1.2	0.1
38E	0	5.0	0	1.3	0.3
25A	0	4.0	0	1.3	0.1
25B	0	3.0	0	1.3	0.1

No. 34A, a lead-containing preparation. It was found that only 1 cent of erythrocytes that had been incubated with this hyaluron were hemolyzed after 60 minutes suspension in 0.30 per cent saline tion at 37°, in contrast to over 99 per cent hemolysis of the co erythrocytes (incubated with normal saline) in the same hypo saline solution.

The amount of potassium released from erythrocytes following the tion of lead depended on the amount of lead added. Table IV set e results of an experiment demonstrating this effect. Similar results ve been obtained with other lead salts; for example, $Pb(NO_3)_2$ with ythrocytes washed and suspended in isotonic $NaNO_3$, $Pb(CH_3COO)_2$ ith erythrocytes washed and suspended in isotonic CH_3COONa, $PbCl_2$ ith erythrocytes washed and suspended in isotonic NaCl.

TABLE IV

Effect of Lead on Potassium Loss from Erythrocytes

5 per cent suspension of normal washed human erythrocytes in isotonic sodium loride solution; incubated for 60 minutes at 37°; Pb^{++} added as $Pb(CH_3COO)_2$.

Pb^{++} added	K^+ in supernatant after incubation	Hemolysis
γ per ml. cells	*m.eq. per l.*	*per cent of cells*
0	0.24	0
5	0.40	0
10	0.83	0
20	4.20	0
40	4.59	0
160	5.20*	0.45

* The loss of all the erythrocyte potassium (equivalent to 100 per cent hemolysis) ould have resulted in a concentration of 5.32 m.eq. of K^+ per liter.

TABLE V

Effect of Addition of Lead Nitrate to Rapidly Sedimenting Blood

To aliquots of 1.5 ml. of oxalated blood were added 0.4 ml. of isotonic $NaNO_3$ ontrol) and 21 γ of Pb^{++} (as $Pb(NO_3)_2$) in 0.4 ml. of isotonic $NaNO_3$ and incubated r 40 minutes at 37°. Blood specimens were then drawn into Westergren sedimention tubes and the fall in erythrocyte columns determined.

	Fall of erythrocyte column, per cent of initial height	
	After 60 min.	After 135 min.
lood + $NaNO_3$ (control)	11	28
“ + $Pb(NO_3)_2$	1	2

Some rapidly sedimenting blood was obtained from a patient with eumatoid arthritis, and lead was added to it. This was to confirm the eory that hyaluronidase preparations affected the ESR by virtue of e lead they contained. Table V clearly shows that the addition of ad lowered the elevated ESR of this blood.

Microscopic examination of erythrocytes which had been incubated plasma containing added lead revealed the typical changes associated ith exposure to this metal. The cells became crenated, then shrunken,

and finally small spheres with smooth or finely crenated surfaces. T
erythrocyte changes were associated with a diminution in hemat
and could not be distinguished from the changes described as t
place after exposure to lead-containing hyaluronidases.

SUMMARY

Of ten preparations of hyaluronidase tested only five were activ
reducing the ESR, and these five preparations contained lead. No
the five inactive preparations contained demonstrable lead. The rel
activity of the various preparations was proportional to their lead
tent. The addition of lead salts to rapidly sedimenting blood, o
erythrocyte suspensions, reproduced all the phenomena associated
the addition of lead-containing hyaluronidases. No evidence was
tained that hyaluronidase preparations had any action on erythro
except by virtue of the lead they contained.

The precise mode of action of lead and other heavy metals in cau
loss of potassium from erythrocytes is subject to current investigatio

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HE EFFECT OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS UPON TYROSINE METABOLISM IN THE SCORBUTIC GUINEA PIG*

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The ingestion of large amounts of aromatic amino acids included in diets ractically devoid of ascorbic acid results in the urinary excretion of several termediary metabolites of these substances in both guinea pigs (1, 2) d premature infants (3). The abnormal constituents of the urine fol- wing tyrosine ingestion have been identified as *p*-hydroxyphenylpyruvic id, *p*-hydroxyphenyllactic acid, and small amounts of tyrosine (1, 3). omogentisic acid has also been recovered from guinea pig urine (1). The egree of hydroxyphenyluria observed seems to depend upon the duration the scorbutigenic régime as well as the amount of tyrosine ingested (2). his apparent defect in tyrosine metabolism disappears following the ad- inistration of small amounts of *l*-ascorbic acid (1, 2, 4).

The failure of *d*-isoascorbic acid to produce the same effect, except in oses 20 times that of the naturally occurring isomer (1), suggests that the itamin C activity of this compound is the essential property involved. his hypothesis is confirmed by the observation of transitory and minimal esponses following the administration of several dicarboxylic acids of the rebs cycle (5) and liver extract (4, 6) as well as the failure of the longer ecognized components of the vitamin B complex to influence the meta- olic aberration.

The reports that pteroylglutamic acid (PGA) increased the oxidation f tyrosine by suspensions of liver from sulfonamide-treated rats (7) and at the high excretion of phenolic compounds by patients having perni- ious anemia in relapse was reduced by liver therapy (8) suggested to us hat PGA might have an effect upon the tyrosine metabolism of the scor- utic guinea pig. Results have been presented demonstrating that PGA bolishes the hydroxyphenyluria of tyrosine-fed guinea pigs on a scorbuti- enic diet (9). This report provides additional details of the methods mployed in these studies and presents further studies showing the prevention of this defect by continuous administration of PGA. In addit the influence of some related substances has been studied.

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* These studies were financed by grants to the Division of Nutrition from the ational Vitamin Foundation, the Nutrition Foundation, Inc., and the Interna- onal Health Division of the Rockefeller Foundation.

Methods

Albino guinea pigs of both sexes weighing approximately 300 gm. w housed in wire bottom cages and fed a scorbutigenic diet of the follow composition: skimmed milk powder heated for 2 hours at 100°, 30; rol oats (fortified), 39; wheat bran, 20; sodium chloride, 1; cod liver oil, and cottonseed oil, 8 per cent. A sample of this diet was found to cont approximately 0.8 γ per gm. of total pteroylglutamates as determined microbiological assay.[1] This diet plus ascorbic acid or cabbage in suita amounts permitted growth and maintained the animals in good condit for periods up to several times the length of those represented by present study. During the experimental periods 5 per cent L-tyros (General Biochemicals) was incorporated in the diet and the animals w kept in metabolism cages of the usual type. Collections of urine w made for serial 24 hour periods in bottles containing 2.5 ml. of 2 N H and a small amount of mineral oil. The urines were diluted to a standa volume of 100 ml., cleared with 1.5 gm. of Lloyd's reagent, and the filtra stored in the cold until analyzed. Control runs demonstrated the stabil of the several aromatic fractions measured under these conditions.

Determinations of total hydroxyphenyl compounds were made by photoelectric adaptation of the method of Folin and Ciocalteu (10) a expressed as tyrosine. Keto acids were estimated by an adaptation of procedure of Friedemann and Haugen (11), and the standardization ma against phenylpyruvic acid. Homogentisic acid was measured by method of Neuberger (12). The results are expressed as per cent of added tyrosine intake for the two preceding 24 hour periods. Ascor acid, when administered, was fed daily by pipette directly into the mou of the animals. PGA and related compounds were injected subcuta ously. The liver extract was given intramuscularly.

EXPERIMENTAL

Four pairs of animals were fed the basal diet plus 5 per cent L-tyros over a 20 day period. Group I served as controls and received no supp ments. Group II was given a supplement of 5 mg. of PGA daily.[2] Gro received 25 mg. of ascorbic acid daily. Group IV received both vita- ns in the dosages indicated above. The average 24 hour excretions of droxyphenyl (as tyrosine) for the 20 day period were 37.3 ± 16.5, ± 4.1, 5.2 ± 1.0, and 4.6 ± 2.2 per cent of added dietary tyrosine spectively for Groups I through IV. Homogentisic acid excretion was nimal for all groups. The data for the individual animals and groups re consistent. Individual charts depicting the data on four of the ani- ls are found in Fig. 1. Additional studies of serum ascorbic acid con- ntrations as influenced by the pteroylglutamates will be considered in a

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[1] This analysis was kindly performed for us by Dr. Paul L. Day of the Departm of Biochemistry, University of Arkansas School of Medicine, Little Rock.

[2] Administered as a solution of folvite (Lederle). We are grateful to Dr. St ton M. Hardy and Dr. Thomas H. Jukes of the Lederle Laboratories Division, Am can Cyanamid Company, for generous supplies of the pteroylglutamates and cr methylfolic acid used in these studies.

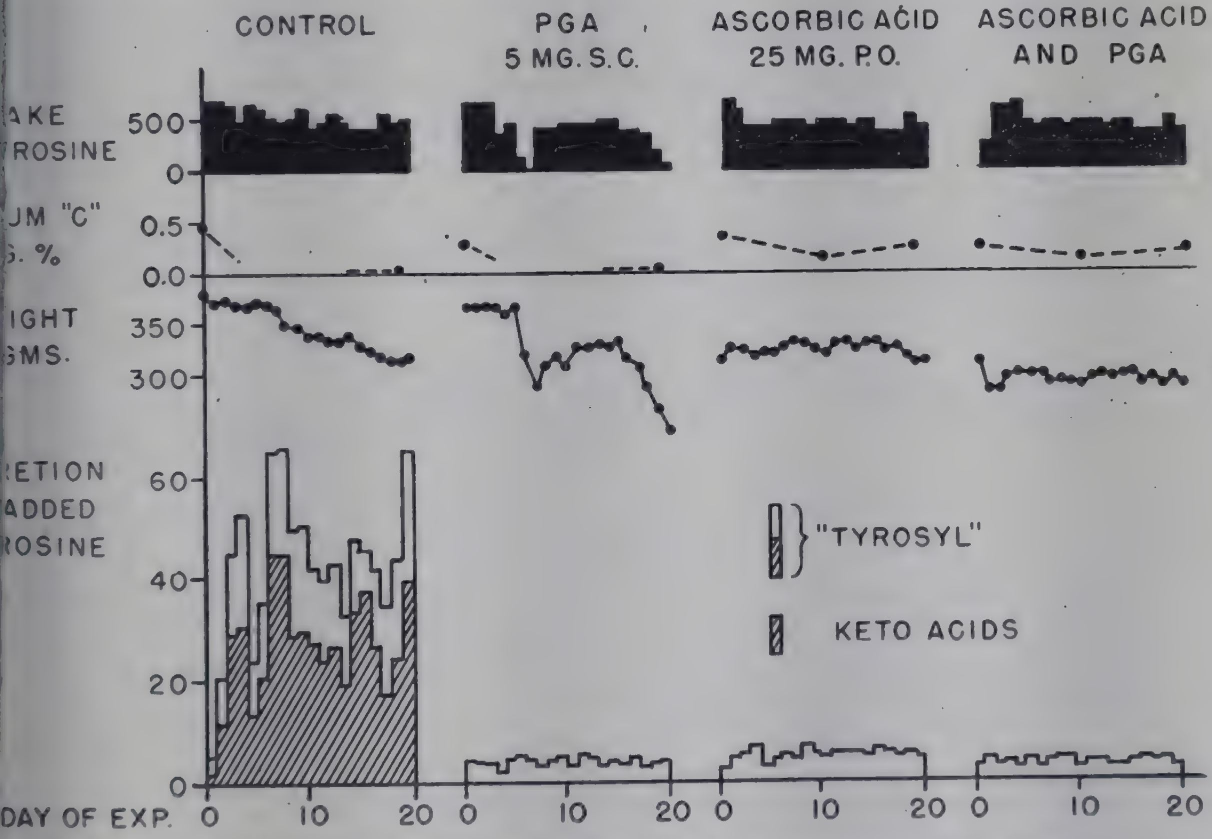

Fig. 1. The effectiveness of pteroylglutamic acid (subcutaneously) and ascorbic id (orally) in the prevention of hydroxyphenyluria in tyrosine-fed guinea pigs. hese data illustrate the daily variability of observations in a typical experiment.

bsequent report.[3] In a second experiment the amount of tyrosine in- ested was equalized by administering it by pipette in aqueous suspension, s was done by Painter and Zilva (2). The animals did not tolerate this rocedure well and consumed but little of the basal diet. Owing to the oor condition of all of the animals the experiment was terminated on the th day of tyrosine supplementation. However, the results presented in able I are comparable to the first experiment. Homogentisic acid excre- on by these animals was not determined. These data confirm the preliminary findings previously reported and show the similar effects of b vitamins.

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[3] Stockell, A. K., Woodruff, C. W., and Darby, W. J., to be published.

Excretions of keto acid and homogentisic acid of less than 1 per cent the added tyrosine are probably not significant. The average excretion hydroxyphenyl compounds for all of the treated animals was 5.4 ± 2 and probably represents the "normal" excretion of these substances un conditions of tyrosine feeding. Quite similar results were obtained Painter and Zilva (2), although they administered glycyltyrosine parent ally in various dosages. Whenever significant hydroxyphenyluria occu an essentially constant ratio of keto acids to total hydroxyphenyl co pounds has been found (2). Our studies confirm this finding. Since p-hydroxyphenylpyruvic acid is also measured quantitatively as hydro phenyl, the more time-consuming keto acid determinations have be omitted in subsequent experiments except for occasional estimations each new situation.

Table I

Effects of Ascorbic Acid and PGA upon Hydroxyphenyluria of Tyrosine-Fed Guinea Pigs Receiving Standard Dose of 700 Mg. Daily by Pipette during 9 day Period

Vitamin supplement	No. of animals	No. of determinations	Average dietary intake	Average excretion per 24 hrs. ±	
				Hydroxyphenyl compounds	Keto acid
			gm. per 24 hrs.	*per cent added tyrosine*	*per cent add tyrosine*
None	3	25	4	24.0 ± 10.7	12.0 ± 7
Ascorbic acid, 25 mg. daily by mouth	2	18	13	9.2 ± 6.2	3.0 ± 3
PGA, 5 mg. daily subcutaneously	3	24	5	6.5 ± 1.9	0.4 ± 0

Five guinea pigs were placed on the scorbutigenic diet containing 5 cent L-tyrosine. After 4 to 7 days on the diet the excretion of hydro phenyl compounds was measured for 2 to 3 days. For the next 4 da each animal was given 5 U. S. P. units of concentrated antiperninci anemia liver extract daily. The excretions were measured during liver extract treatment and for 2 or 3 days after the completion of injections. The results are reported in Table II. No significant decre in hydroxyphenyluria was noted during the treatment period. Hom gentisic acid determinations were not made. This batch of liver extr had been assayed clinically.[4] Obviously 5 units of active antipernici anemia liver extract did not alter the hydroxyphenyluria.

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[4] We wish to thank Dr. Thomas H. Jukes of the Lederle Laboratories Divis for making this information available to us.

The action of two synthetic conjugates of PGA in preventing the defect as been studied. Pteroyldiglutamic acid and pteroyltriglutamic acid[5] in oses equivalent to 5 mg. of PGA were given daily to two pairs of guinea igs receiving the basal diet containing 5 per cent L-tyrosine. Another air served as controls. These animals were not albinos. The excretion f homogentisic acid and hydroxyphenyl compounds between the 11th and 6th days on the diet is reported in Table III. These days coincided with the greatest excretion of metabolites by the control animals. The effect of the triglutamate was comparable to that of PGA, while the diglutamate had no apparent effect upon tyrosine metabolism under these conditions.

TABLE II

Effect of Daily Intramuscular Injection of 5 Units of Purified Liver Extract upon Hydroxyphenyluria of Five Tyrosine-Fed Guinea Pigs

Period	No. of determinations	Average tyrosine intake	Average 24 hr. excretion of hydroxyphenyl compounds ± S.D.
		mg. per 24 hrs.	*per cent added tyrosine*
Before treatment, 2 or 3 days	12	486	20.9 ± 14.0
During " 4 days	20	507	24.9 ± 14.4
After " 2 or 3 days	13	411	25.9 ± 14.6

TABLE III

Effects of PGA Conjugates on Hydroxyphenyluria of Tyrosine-Fed Guinea Pigs during 6 Day Period

The results represent twelve determinations on two animals.

Vitamin supplement*	Average tyrosine intake	Average excretion per 24 hrs. ± S.D.	
		Hydroxyphenyl compounds	Homogentisic acid
	mg. per 24 hrs.	*per cent added tyrosine*	*per cent added tyrosine*
None	504	17.3 ± 12.0	0.6 ± 0.14
Pteroyldiglutamic acid subcutaneously	571	29.9 ± 15.7	0.5 ± 0.27
Pteroyltriglutamic " "	604	3.7 ± 1.0	3.8 ± 1.98

* Administered in doses equivalent to 5 mg. of PGA daily.

Although Sealock and Silberstein (1) identified homogentisic acid in the urine of guinea pigs excreting hydroxyphenyl compounds under conditions similar to those employed in the present study, Painter and Zilva (2) failed to confirm this observation. The inconstant presence of alkaptonuria in

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[5] Administered as diopterin and teropterin (Lederle), respectively.

the experiments here reported and its occurrence only in the absenc
significant hydroxyphenyluria suggested that the excretion of homogent
acid by tyrosine-fed guinea pigs may not be related to this apparent de
in tyrosine metabolism. We have attempted to produce a deficiency
PGA in several animals by including a metabolic antagonist, crude met
folic acid (13), in the basal diet at a level of 1 and later 2 per cent, sup
menting this diet with 25 mg. of ascorbic acid daily and 5 per cen
tyrosine as in the previous experiments. The one surviving animal a
days was still gaining weight and had normal hematological findi
There was no hydroxyphenyluria. Homogentisic acid excretion[6] reac
a level of 10 per cent of the added tyrosine appeared at about the 3rd we
Treatment with 5 mg. of PGA daily for 1 week failed to alter the ho
gentisic acid excretion. The appearance of alkaptonuria during asco
acid ingestion and its failure to disappear following the administratio
large amounts of PGA would also suggest that some other mechanisr
involved in its production. Further investigations of this subject are
in progress.

DISCUSSION

These experiments show that PGA will both prevent and abolish
hydroxyphenyluria seen in guinea pigs fed diets containing large amou
of tyrosine and devoid of vitamin C. Similarly, pteroyltriglutamic
will prevent its development. However, in the two animals studied,
diglutamic derivative was without effect. In curative experiments
ploying a liver extract of known antipernicious anemia potency, no sig
cant effect was found. Sealock and Lepow (6), who employed sligh
different dietary conditions and measured the keto acid rather than
total hydroxyphenyl excretion, used larger amounts of liver extract. H
ever, the moderate reduction in keto acid excretion which they have
ported is considerably less than that which we find after 5 mg. of P
This difference in results may be a manifestation of the qualitative dif
ence between PGA and the antipernicious anemia substances presen
liver extract as well as the difference in dosage. Studies on the effec
vitamin B_{12} on hydroxyphenyluria are in progress.

Several examples suggesting an interrelationship between ascorbic
deficiency and megaloblastic anemias may be found in the literat
This subject has recently been discussed by R. W. Vilter (14) who c
two cases of pernicious anemia from the British literature which appea
to be unresponsive to liver therapy until ascorbic acid was administe
The experience of Dyke *et al.* (15) is especially interesting because a rev

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[6] We are indebted to Dr. H. B. Lewis for the sample of homogentisic acid use
the standardization of our method.

all their cases of pernicious anemia maintained on liver therapy showed slight, temporary reduction in red cell counts simultaneously with an crease in clinical scurvy in the British Isles (16) during the spring of 42. The anemia seen in patients with clinical scurvy is occasionally acrocytic in type and often improves on hospital diets low in vitamin C thout specific treatment (17). In six of twelve patients with nutritional acrocytic anemia or pernicious anemia in relapse, showing biochemical idence of vitamin C deficiency, the daily administration of 500 to 1000 g. of ascorbic acid was followed by reticulocyte responses (18). Recent vestigations of megaloblastic anemias in infants have revealed a rearkably frequent occurrence of diets practically devoid of vitamin C nong the anemic children. Eleven of the twenty-five patients reported Zuelzer and Ogden (19) and all of the fourteen patients receiving a simuted breast milk reported by May (20) had partaken of diets quite low in corbic acid. The observations on tyrosine metabolism in pernicious emia which suggested the present study constitute another example om investigations on the human which strongly indicate a possible metalic interrelationship between ascorbic acid and the hemapoietic vitamins.

The effect of PGA upon this apparent defect in tyrosine metabolism in emature infants has recently been studied (21). In four of ten infants veloping hydroxyphenyluria while receiving high protein diets devoid of tamin C, the administration of 5 to 30 mg. of PGA daily resulted in a minution in the excretion of these compounds. Subcutaneous injection this vitamin was found to be more effective than oral administration, a nding in keeping with our experience in guinea pigs (9).

The observations of Johnson and Dana (22) that scorbutic symptoms opeared in rats maintained on a PGA-deficient diet and that a response llowed ascorbic acid administration seemed to provide further experiental evidence of an interrelationship between these two vitamins. Johnn[7] has indicated, however, that this effect may not be a specific one, but at it is more probably related to the quantity of food consumed by the imals.

SUMMARY

1. The hydroxyphenyluria produced in guinea pigs fed scorbutigenic ets containing 5 per cent L-tyrosine is prevented by both PGA and its iglutamic homologue as well as by ascorbic acid. These pteroylglumates do not protect the guinea pig against scurvy.

2. Neither pteroyldiglutamic acid nor liver extract in the amounts used ad a demonstrable effect upon the hydroxyphenyluria.

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[7] Johnson, B. C., personal communication.

3. Observations upon the excretion of homogentisic acid un conditions are reported.

4. Possible interrelationships between the metabolic rôles of ascorbic acid are discussed.

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METABOLIC STUDIES ON T_2 ESCHERICHIA COLI BACTERIOPHAGE

TUDY OF DESOXYPYRIDOXINE INHIBITION AND ITS REVERSAL

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part of the studies on chemotherapeutic agents capable of combating infections, we have investigated the effect of certain chemical com- s on the multiplication of bacteriophage. It was hoped that infor- n obtained might be applied to studies on viruses in animals.

ny compounds were studied for their effect on the multiplication of T_2 *ichia coli* bacteriophage. Of those tested, it was found that desoxy- xine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine[1]) markedly red with the multiplication of the bacteriophage without materially ng the rate of growth of the bacteria.

Materials and Methods

T_2r^- bacteriophage[2] was used in these studies. This phage has a utation rate to a type giving larger, smooth types of plaques. Such ts have different nutritional requirements than the r^- wild type. To in the r^- type, it was necessary either to go back to the dried l culture or frequently to pick from plate cultures plaques show- e desired characteristics.

stock bacteriophage was prepared by adding approximately 10^7 *ichia coli* cells from a 4 hour broth culture and 10^8 bacteriophage es to each ml. of tryptose phosphate broth. The cultures were ted at 37° for 4 hours and filtered. This filtrate, which contained 10^{10} phage particles, was stored at 5°. The culture of *Escherichia* s maintained by transfers to nutrient agar slants each week.

experimental medium contained 0.5 per cent acid-hydrolyzed casein, cent glucose, 10 mg. per cent cystine, 1 mg. per cent each of adenine , guanine hydrochloride, and uracil, and 0.25 per cent each of mineral and B (1). The medium and the compounds to be studied were d to pH 7.2 with sodium hydroxide.

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are indebted to Merck and Company, Inc., Rahway, New Jersey, for sup- s with generous portions of this compound.

eived through the courtesy of Dr. M. R. Zelle, formerly of the National In- of Health.

Test-tubes were set up containing 2 ml. of the medium and the c pounds to be studied. These were sterilized at 120° for 10 minutes steam pressure autoclave. The *Escherichia coli* suspensions were prep by inoculating tryptose phosphate broth from a recent agar slant cult This was incubated for 18 hours. From this a 4 hour broth culture made. The cells were washed twice with 0.85 per cent NaCl solution brought to the original volume. 0.1 ml. of bacteriophage suspension c taining approximately 1×10^3 phage particles and 0.1 ml. of the *Escheri coli* suspension was added to each ml. of the test medium. The tubes v incubated for the desired length of time. Plaque counts were made the two-layer plate method of Hershey *et al.* (2).

TABLE I

Effect of Desoxypyridoxine on Multiplication of Escherichia coli and of T_2 Bacteriophage

Five tests each.

	After 3 hrs.		After 5 hrs.	
	Photometer*	Plaques†	Photometer*	Plaques†
Control	71	4.4×10^8	61	1.6×1
	73	(9.4×10^8)	66	$(4.8 \times 1$
	69	(1.7×10^8)	57	$(2.2 \times 1$
Desoxypyridoxine, 0.5 mg. per ml.	74	3.7×10^6	68	1.9×1
	76	(5.6×10^6)	71	$(5.1 \times 1$
	72	(1.0×10^6)	64	$(2.1 \times 1$

The figures in parentheses indicate the range.

* Evelyn photometer readings; 100 = no growth.

† Plaque counts of phage particles.

Evelyn colorimeter tubes containing 10 ml. of the medium and c pounds to be tested, in amounts per ml. equal to the contents of the sma tubes, were prepared and sterilized. The tubes were inoculated *Escherichia coli*, incubated, and read turbidimetrically.

Results

The data presented represent several tests with each of the substan Tables I to VI give average values and ranges encountered in the var experiments. The effect of desoxypyridoxine on the multiplication o bacteriophage is shown in Table I. The plaque counts at 0 and aft hour's incubation were similar in both tubes. After 3 and 5 hou marked difference in phage production was noted. The number of pl rticles in the tubes containing the desoxypyridoxine was approximately 1 r cent of that in the controls at the end of the 5 hour incubation. The cterial growth in the tubes inoculated with *Escherichia coli* alone showed ry little difference whether desoxypyridoxine was in the medium or not. five tests the colorimeter readings of the control tubes after 5 hours cubation ranged from 57 to 66, while those containing the drug ranged om 64 to 71. It is also shown in Table II that desoxypyridoxine had tle effect on the number of viable bacteria at any period of exposure om 0 to 5 hours at 37°.

The inhibiting effect of desoxypyridoxine on phage production was comletely neutralized by pyridoxine when as little as 0.01 mg. per ml. was added to tests containing 0.5 mg. of the vitamin analogue (Table III). Smaller amounts of pyridoxine were not tested.

TABLE II

ffect of Desoxypyridoxine on Growth of Escherichia coli Determined by Plate Count

Test No.		Duration of incubation		
		0	1 hr.	2 hrs.
1	Control	2.1×10^7	1.6×10^7	4.0×10^7
	Desoxypyridoxine, 1 mg. per ml.	1.7×10^7	3.0×10^7	2.0×10^7
2	Control	2.1×10^6	9.9×10^6	1.8×10^7
	Desoxypyridoxine, 1 mg. per ml.	2.0×10^6	1.1×10^7	1.9×10^7
		3 hrs.	4 hrs.	5 hrs.
1	Control	1.1×10^8	2.1×10^8	2.9×10^8
	Desoxypyridoxine, 1 mg. per ml.	1.5×10^8	1.5×10^8	2.0×10^8
2	Control	5.4×10^7	1.9×10^8	2.4×10^8
	Desoxypyridoxine, 1 mg. per ml.	5.2×10^7	1.5×10^8	1.5×10^8

Many compounds were studied for their influence on the drug inhibition of the phage. Some members of two general classes of compounds were ound to neutralize the inhibition of the phage production. These were atty acids and intermediates of carbohydrate metabolism.

Fatty Acids—The effect of fatty acids on desoxypyridoxine inhibition of hage is shown in Table IV. 5 mg. of formic, acetic, butyric, or valeric acid completely neutralized the inhibition caused by 0.5 mg. of desoxypyridoxine. 5 mg. of propionic acid, which markedly inhibited the growth of the bacteria, gave irregular production of the virus in both the control tubes and in those containing desoxypyridoxine. Acetic or valeric acids ncreased phage growth in the presence of the inhibitor, even though the bacterial growth was retarded by these fatty acids considerably below the levels obtained with desoxypyridoxine alone. Caproic and capryl each retarded the growth of both bacteria and phage. When pyridoxine was included with these latter compounds, phage pro was not further decreased. This might indicate that the inhibiti also neutralized by these compounds. Tests were made to det whether the growth stimulation of the phage by acetic acid in the p of the inhibitor was the result of a buffering effect towards a high The results shown in Table V indicate no significant difference in values for the control or desoxypyridoxine tubes.

TABLE III

Effect of Pyridoxine on Desoxypyridoxine Inhibition of T_2 Bacteriophag Multiplication

	No. of tests	Control		Desoxypyrid 0.5 mg. pe	
		Photometer*	Plaque counts†	Photometer*	Plaqu
No additions	5	66	2.0×10^9	69	1.8
		71	(4.8×10^9)	75	(2.6
		60	(2.2×10^8)	64	(1.1
Pyridoxine, 0.5 mg. per ml.	5	65	8.5×10^8	63	2.7
		69	(2.6×10^9)	69	(8.0
		59	(1.7×10^8)	55	(2.0
" 0.1 " " "	3	68	6.9×10^8	67	5.0
		70	(1.5×10^9)	72	(6.5
		66	(2.6×10^8)	63	(2.0
" 0.01 " " "	3	69	2.0×10^8	68	2.8
		70	(3.5×10^8)	75	(4.4
		66	(8.4×10^7)	63	(2.1

The figures in parentheses indicate the range.

* Turbidimetric readings of bacterial growth after 5 hours incubation (photometer).

† Plaque counts of phage particles after 5 hours incubation.

Carbohydrate Intermediates—The effect of intermediates of carboh metabolism on the desoxypyridoxine inhibition of phage multiplic shown in Table VI. The addition of 2.5 mg. per ml. of glucose medium had no effect on virus multiplication. In tests not sh Table VI, concentrations up to 10 mg. per ml. also had no effect. 2.5 mg. per ml. of glucose-6-phosphate or pyruvic acid were added tubes containing the inhibitor, maximum growth of the virus occ When similar amounts of lactic, malic, fumaric, or succinic acid loyed, some inhibition reversal was recorded, but they were not as tive as the two former carbohydrate intermediates.

TABLE IV

Effect of Fatty Acids on Desoxypyridoxine Inhibition of T_2 Bacteriophage Multiplication

dition, 5.0 mg. per ml.	No. of tests	Controls		Desoxypyridoxine, 0.5 mg. per ml.	
		Photometer*	Plaque counts†	Photometer*	Plaque counts†
additions	3	65	1.3×10^9	67	3.9×10^7
		67	(2.1×10^9)	70	(5.1×10^7)
		63	(6.8×10^8)	64	(5.4×10^6)
mic acid	2	73	4.4×10^9	70	2.6×10^9
		74	(5.8×10^9)	71	(3.3×10^9)
		73	(2.9×10^9)	70	(1.9×10^9)
tic "	3	78	2.1×10^9	76	2.8×10^9
		78	(2.9×10^9)	78	(5.2×10^9)
		78	(1.0×10^9)	72	(1.5×10^9)
pionic acid	3	92	1.0×10^9	95	4.5×10^8
		96	(2.9×10^9)	97	(1.2×10^9)
		85	(6.3×10^7)	94	(3.1×10^7)
utyric "	3	69	1.6×10^9	67	1.4×10^9
		71	(2.7×10^9)	72	(3.0×10^9)
		66	(9.8×10^8)	62	(3.3×10^8)
aleric "	3	79	1.0×10^9	79	1.9×10^9
		80	(2.1×10^9)	80	(5.0×10^9)
		77	(3.5×10^8)	76	(2.5×10^8)
proic acid	3	87	1.1×10^7	86	1.5×10^7
		88	(2.0×10^7)	86	(2.7×10^7)
		85	(4.0×10^6)	86	(9.9×10^6)
prylic "	3	84	5.0×10^7	84	7.0×10^6
		86	(5.0×10^7)	84	(1.5×10^7)
		82	(5.0×10^7)	83	(1.0×10^6)

The figures in parentheses indicate the range.

* Turbidimetric readings of bacterial growth after 5 hours incubation (Evelyn otometer).

† Plaque counts of phage particles after 5 hours incubation.

DISCUSSION

Spizizen (3) found that certain metabolic poisons, *viz.* cyanide, iodoace-te, arsenite, 2,4-dinitrophenol, and *p*-aminophenol, in low concentra-ons inhibited the multiplication of "anti-*Escherichia coli* bacteriophage ." His data seem to indicate a rather narrow range between the dose

TABLE V

Effect of Acetic Acid on pH of Medium after Incubation with Phage ar
Escherichia coli

Test No.		After 3 hrs.		After 5	
		pH	Plaque counts	pH	Plaq
1	Control	6.54	1.0×10^8	6.5	2.5
	" + acetic acid, 5 mg. per ml.	6.59	4.5×10^8	6.51	3.0
	Desoxypyridoxine, 1 mg. per ml.	6.4	2.3×10^6	6.45	9.4
	" + acetic acid	6.48	2.2×10^8	6.49	1.8
2	Control	6.51	1.5×10^7	6.58	2.9
	" + acetic acid, 5 mg. per ml.	6.5	1.5×10^8	6.52	2.2
	Desoxypyridoxine, 1 mg. per ml.	6.38	5.4×10^5	6.38	3.7
	" + acetic acid	6.39	2.1×10^7	6.48	1.1

TABLE VI

Effect of Carbohydrate Intermediates on Desoxypyridoxine Inhibition of
Bacteriophage Multiplication

Additions, 2.5 mg. per ml.	No. of tests	Plaque counts*	
		Controls	Desoxypyri 0.5 mg. pe
No additions	8	2.8×10^9	$3.1 \times$
		(6.0×10^9)	$(1.3 \times$
		(4.6×10^8)	$(1.6 \times$
Glucose	3	9.4×10^8	$7.4 \times$
		(1.9×10^9)	$(1.4 \times$
		(4.3×10^8)	$(2.6 \times$
Glucose-6-phosphate	4	3.4×10^9	$2.5 \times$
		(5.8×10^9)	$(5.8 \times$
		(1.9×10^9)	$(1.9 \times$
Pyruvic acid	4	6.5×10^9	$2.9 \times$
		(9.1×10^9)	$(6.4 \times$
		(4.5×10^9)	$(9.9 \times$
Lactic "	4	6.0×10^9	$1.1 \times$
		(1.0×10^{10})	$(2.9 \times$
		(3.9×10^8)	$(9.4 \times$
Malic "	4	4.4×10^9	$7.2 \times$
		(7.2×10^9)	$(1.7 \times$
		(2.5×10^9)	$(2.0 \times$
Fumaric "	4	4.6×10^9	$1.3 \times$
		(9.6×10^9)	$(2.2 \times$
		(6.1×10^8)	$(6.4 \times$
Succinic "	4	3.9×10^9	$3.0 \times$
		(7.5×10^9)	$(7.3 \times$
		(1.6×10^9)	$(6.1 \times$

The figures in parentheses indicate the range.

* Plaque counts of phage particles after 5 hours incubation.

inhibited the phage and the dose which inhibited the multiplication host cells. Cohen and Anderson (4) found that 5-methyltryptophan ited the multiplication of *Escherichia coli* B without affecting the rate ygen utilization or the R.Q. in a synthetic medium. Bacteria in- with T_2 or T_4 bacteriophage, in the presence of the compound, were e to reproduce the virus. Fitzgerald and Lee (5) inhibited lysis of -infected *Escherichia coli* cells with several acridine compounds and eracted the antiviral effect with ribonucleic acid.

have shown that desoxypyridoxine, an analogue of pyridoxine that ecific antivitamin activity in animals (6), inhibits the multiplication *scherichia coli* bacteriophage without materially affecting the growth *Escherichia coli*. This inhibition can be neutralized by pyridoxine. alogy to other work with vitamin-antivitamin relationships in bac- it might be assumed that pyridoxine is necessary for the multipli- of this phage, and that desoxypyridoxine inhibits the multiplication competitive action against the vitamin.

mechanism by which certain carbohydrate intermediates reversed pyridoxine inhibition would not seem to be so direct. Since glucose not neutralize the inhibition but glucose-6-phosphate or pyruvate , it seems possible that desoxypyridoxine may act either directly or h pyridoxine inhibition to interfere, in some manner, with glucose tion. Whether the ability of formic, acetic, butyric, and valeric o reverse the desoxypyridoxine inhibition is related to the above is ident at this time.

SUMMARY

oxypyridoxine, 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine, ed the multiplication of T_2r^+ *Escherichia coli* bacteriophage under ions that otherwise produced near maximum virus production. The pyridoxine-bacteriophage inhibition was reversed not only by pyri- , but also by several fatty acids and glucose-6-phosphate, pyruvic nd somewhat less effectively by lactic, malic, fumaric, or succinic

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THE FAILURE TO UTILIZE α-AMINOADIPIC ACID AS A PRECURSOR OF LYSINE BY RATS AND CERTAIN BACTERIA*

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orsook, Deasy, Haagen-Smit, Keighley, and Lowy (1) have recently ribed a new amino acid, α-aminoadipic acid, as an intermediary luct of lysine metabolism. Subsequently Mitchell and Houlahan reported that this amino acid can function as a precursor of lysine *Neurospora* mutant. The present experiments were first under- n to determine, at the suggestion of Dr. H. Borsook, whether rats d similarly utilize this newly discovered amino acid as a precursor ysine; furthermore, they were made to determine whether this amino influences the growth of bacteria commonly used for microbiological ys.

Experiments on Rats

the first experiment an attempt was made to maintain the growth of ntile rats when a portion of the lysine of the diet was replaced by ninoadipic acid. Male Sprague-Dawley rats were used in three ıps of six rats each. The technique of paired feedings was used ughout. The composition of the diets is shown in Table I.

able II shows that rats of the first group, which received only the ll amount of lysine present in gluten (Diet 1), failed to grow, while in the second group with an adequate L-lysine supplement (Diet 2) satisfactorily. Animals in the third group, receiving equivalent unts of the aminoadipic acid (Diet 3) instead of the lysine, did not v.[1] Racemic and optically active α-aminoadipic acids gave the same tive results.

hese experiments indicate that the growing rat cannot utilize this no acid as a precursor of lysine.

he rats receiving α-aminoadipic acid developed a serious diarrhea in 1 or 2 days. We, therefore, investigated the possibility that

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The expenses of this study were in part defrayed by a grant from the National min Foundation.

Histological investigation of the kidneys and livers of these rats did not reveal pathological changes.

TABLE I

Composition of Diets

Diet No.	Wheat gluten	Tryptophan	α-Amino-adipic acid	L-Lysine HCl	Casein	Basal
1	11.0	0.1				89
2	11.0	0.1		1.2		87
3	11.0	0.1	2.4			85
4					13.0	87
5			1.5		13.0	85

* Corn-starch 3050 gm. (78.3 per cent), rice bran concentrate 400 gm. (10. cent); cottonseed oil 200 gm. (5.13 per cent), U. S. P. salt mixture 200 gm. (5. cent), fish oil (1 gm. contains 2000 I.U. of vitamin A and 400 I.U. of vitamin gm. (1.27 per cent), riboflavin 75 mg., Ca pantothenate 150 mg., and choline ch 2.5 gm.

TABLE II

Growth of Rats in Gm. for 16 Day Period on Diets 1 to 5

Diet	Weight of rats		Total gain	Averag food co tion p
	At start of experiment	At end of experiment		
			gm.	*g*
1. Gluten	81	87	6	14
	91	95	4	
	77	75	−2	
	82	84	2	
	81	84	3	
	78	79	1	
2. Gluten + lysine	83	101	18	1
	79	109	30	
	82	115	33	
	77	111	34	
	92	123	31	
	84	112	28	
3. Gluten + α-aminoadipic acid	89	90	1	1
	81	77	−4	
	82	88	6	
	77	74	−3	
	93	92	−1	
	87	87	0	
4. Casein	57	91	34	1
	41	71	30	
	55	94	39	
5. Casein + α-aminoadipic acid	62	93	31	1
	45	88	43	
	47	86	39	
	57	92	35	
	53	85	32	
	51	91	40	

diarrhea might influence the normal growth of the animals. We pared the growth of rats on a standard diet (Diet 4) with the growth nimals on the same diet with α-aminoadipic acid added in the quanti- of 0.5 per cent, 1 per cent, and 1.5 per cent (Diet 5). Those rats ch received the amino acid supplement showed a normal growth rate ing a 16 day period in spite of severe diarrhea. The mechanism of diarrhea will be investigated further.

Experiments with Bacteria

t was of practical importance to ascertain whether bacteria could ize this amino acid as a precursor of lysine, and whether its presence erferes with the microbiological assay of lysine. The microbiological ay technique of Dunn *et al.* (3) was employed.

xperiments were carried out with *Lactobacillus delbrueckii* LD5, *L. fer- nti*, *L. casei*, and *L. arabinosus* 17-5, which do not require lysine. No nge of growth occurred if large amounts (104 γ per ml.) of α-amino- pic acid were added to the media.

euconostoc mesenteroides and *Streptococcus faecalis*, which require ine, have been investigated and it was found that the addition of α- inoadipic acid to the basal medium did not promote growth. The acid duction on different concentrations of lysine was unchanged by addi- n of various amounts of α-aminoadipic acid. These results indicate t, unlike the *Neurospora* mutant, *Leuconostoc mesenteroides* and *Strep- occus faecalis* cannot utilize α-aminoadipic acid, and that the presence α-aminoadipic acid in the medium does not interfere with the micro- logical determination of lysine.

We are indebted to Dr. H. Borsook and to Dr. A. J. Haagen-Smit the samples of α-aminoadipic acid used in these experiments.

SUMMARY

It has been shown that neither rats nor those bacteria commonly used microbiological assays are able to utilize α-aminoadipic acid as a ecursor of lysine.

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HE PURIFICATION AND PROPERTIES OF A TRIACETIC ACID-HYDROLYZING ENZYME*

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(Received for publication, November 15, 1948)

a previous paper from this laboratory (1) it was shown that triacetic (β,δ-diketohexanoic acid) is split by liver homogenates to acetoacetic acetic acids. Brief reference was also made to a 100-fold purified en-e which catalyzed the above hydrolysis. The object of this paper is lescribe the preparation of this enzyme from beef liver, to record some ts properties, and to present some of the kinetics of the catalyzed re-on.

EXPERIMENTAL

Ianometric Assay—This test is based on the formation of an extra mole cid during the reaction

$$\underset{\text{Triacetic acid}}{CH_3COCH_2COCH_2COOH} \rightarrow \underset{\text{Acetoacetic acid}}{CH_3COCH_2COOH} + \underset{\text{Acetic acid}}{CH_3COOH}$$

sing the liberation of CO_2 from a bicarbonate medium. The test was ried out in conventional Warburg flasks with a volume of reagents total-3.3 ml. The main compartments contained 0.45 ml. of 0.15 M $NaHCO_3$,) ml. of appropriately diluted enzyme, and distilled water to make a ume of 2.70 ml. The side arms contained 0.50 ml. of 0.02 M triacetic d[1] (freshly dissolved and neutralized with NaOH), and 0.10 ml. of 0.15 M HCO_3. A gas mixture containing 95 per cent N_2-5 per cent CO_2 was sed through the vessels for 5 minutes, the stop-cocks closed, and tem-ature equilibrium (30°) reached. After dumping the side arm solu-ns, readings were taken at 5 minute intervals for 30 minutes or until ne 150 μl. of the theoretical 224 μl. of CO_2 were liberated. As illustrated Fig. 1, there was a linear relation between CO_2 production and time, and portionality between rate and enzyme concentration. In general, the nometric procedure was used with purified enzyme preparations where

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[*] This research was supported by grants from the Nutrition Foundation, Inc., Sugar Research Foundation, Inc., and the Division of Research Grants and lowships of the National Institutes of Health, United States Public Health vice.

[1] The authors are indebted to Dr. Robert F. Witter for the triacetic acid used in se experiments.

there was no appreciable "CO_2 retention" due to the protein p
When the error was significant, a correction factor was applied. T
lidity of the correction was confirmed by direct analysis of the triacet
in the flask.

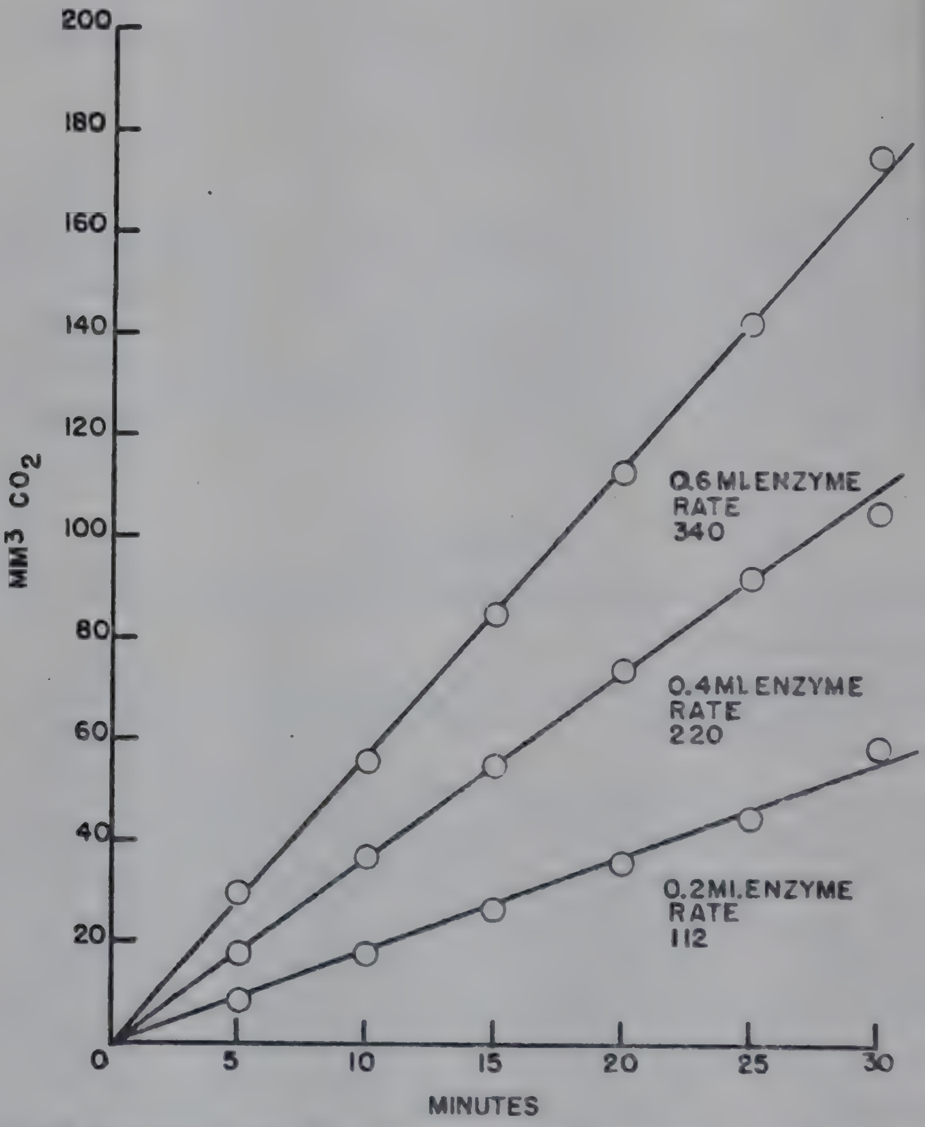

FIG. 1. The proportionality between enzyme concentration and acid pro
in the catalyzed triacetic acid hydrolysis. The experiments were carried
Warburg flasks with bicarbonate buffer; gas phase 95 per cent N_2-5 per ce
temperature 30°.

Colorimetric Test System—For experiments in which the pH and su
concentrations were varied, the incubation was carried out in 15 ml
at a volume of 1.65 ml. Maleate buffer (2, 3) was generally employe
a Warburg apparatus was used for shaking and temperature contro
At the end of a given incubation period, the reaction was stopped by
6 ml. of 5 per cent metaphosphoric acid, and the final volume brou
15 ml. with distilled water. After centrifugation, aliquots of the

nt fluid were analyzed for the triacetic acid remaining, by the method Vitter, Snyder, and Stotz (4). In this case, also, a linear relation was ined between triacetic acid disappearance and enzyme concentration. en lower concentrations of substrate were used, the volume of the cenuged supernatant was kept low by the addition of more concentrated aphosphoric acid and the triacetic acid method scaled down to smaller umes for the desired sensitivity.

or expressing the purity of the enzyme, a dry weight determination 0°) of the preparation was made after thorough water dialysis. At some ges of the purification the photometric measurement of turbidity with fosalicylic acid proved to be a rapid and convenient measure of the pron present.

Enzyme Units and Purity—1 unit of enzyme has been designated as that ount which, under the conditions of the manometric test described sed the liberation of 1 μl. of CO_2 per hour. Purity is expressed as units enzyme per mg. of dry weight of the dialyzed preparation.

Purification of Enzyme

The preparation of the enzyme purified 100-fold over the liver homonate was accomplished essentially by a combination of alcohol fractionan, heat denaturation, and acetone precipitation steps. Details of the ocedure with recoveries and purity at intermediate stages follow.

Stage I. Preliminary Treatment of Beef Liver—One fresh beef liver (7 to pounds) was taken directly from the slaughter-house in an iced coniner to a cold room (0–5°). The liver was cut into sections and passed rough a meat grinder. A 200 gm. portion was supended in 400 ml. of -cold 0.85 per cent NaCl in a Waring blendor jar, and homogenized for 2 inutes.

The combined homogenates from 1800 gm. of tissue (nine runs) were alwed to stand 3 to 5 hours in the cold room. Volume 5260 ml.; total its 596,000; purity 1.66 units per mg. of dry solids.

Stage II. Precipitation and Concentration of Enzyme—To the homogene (5260 ml.) were added 5844 ml. of ice-cold 95 per cent ethyl alcohol, th stirring, to produce a final concentration of 50 per cent alcohol. The ixture was allowed to stand at 4° for 7 hours with occasional stirring. It as then filtered (fluted Schleicher and Schüll papers, No. 595), the funnels ing covered with large watch-glasses to minimize evaporation.

To the 7220 ml. of clear amber filtrate were added, with stirring, 5776 ml. 95 per cent ice-cold ethyl alcohol to bring the alcohol concentration to per cent. After permitting 7 hours standing at 4° for the precipitation of e enzyme, the supernatant fluid was siphoned from the precipitated proins and the latter compacted by centrifugation in the cold. The precipi-

tate was then dissolved by adding cold 0.85 per cent NaCl soluti
total volume of 150 ml., and the mixture stirred and allowed to sta
to 1 hour for maximum solution. The material was centrifuged in t
the supernatant saved, and the salt extraction procedure repeatec
remaining precipitate. The supernatant fluids from the salt ext
were then combined. Volume 270 ml.; total units 146,000; recover
cent; purity 33 units per mg.

With smaller scale preparations, with which it was technically
centrifuge all of the 70 per cent alcohol precipitate rather than siph
described above, it was possible, since much of the precipitate w
dispersed, to obtain approximately double the yield of enzyme
above.

Stage III. Selective Heat Denaturation—270 ml. of solution fro
II were placed in a 500 ml. Erlenmeyer flask and a thermometer in
the solution. The flask was immersed in a water bath at appro
60° and the flask contents maintained at a temperature of 50° for 5
with continual stirring. The flask was then promptly cooled in ic
and the voluminous precipitate of denatured proteins centrifuged
carded. Volume 232 ml.; total units 109,000; recovery 18 per cent
82 units per mg.

Stage IV. Acetone Fractionation—To the 232 ml. from Stage
ml. of ice-cold redistilled acetone were added with stirring, making
concentration 35 per cent acetone. The mixture was allowed to
hour at 4°, after which time the precipitated proteins were removec
trifuging in the cold. The 350 ml. of clear supernatant fluid w
made to 40 per cent acetone by adding 30 ml. of ice-cold acetone
ring. After standing again for 1 hour, the mixture was centrifu
this time the supernatant fluid was discarded. The precipitate
solved in approximately 60 ml. of cold 0.85 per cent NaCl, and
amount of insoluble material filtered off. The clear light green
constituted our purest preparation. Volume 58 ml.; total units
recovery 11 per cent; purity 171 units per mg.

Ammonium sulfate and alcohol fractionations of Stage III pre
also produced purifications similar to acetone, but usually with
coveries.

Properties of Enzyme

Products of Reaction and Specificity—In a previous paper (1) it w
that the products of the catalyzed triacetic acid hydrolysis we
acetic and acetic acids. The purified enzyme had no effect on
ester, triacetic lactone, acetylacetone, or acetoacetic acid as deter
direct analysis for these substrates. As determined by failure to

acid manometrically, β-ketohexanoic acid, oxalacetic acid, α-keto-
ic acid, levulinic acid, sorbic acid, and hexanoic acid were not at-
l.

er completion of our work, the paper of Meister and Greenstein (5)
red which described the preparation of a 10-fold purified enzyme
lpyruvase") capable of hydrolyzing α,γ-diketo acids. Because of
reat structural similarity of these acids to triacetic acid, 2,4-diketo-
ioic acid was synthesized (6, 7) and tested with our enzyme. It was
lyzed with great rapidity, and it is therefore possible that the two en-
s are identical. Both enzymes could be classified as diketo acid des-
ses; a more specific term for our enzyme is not suggested at this time.

bility of Enzyme—The enzyme preparation resulting from Stage III
be stored frozen for at least 3 months without loss of activity, and at
r 1 month. The more purified preparation appeared less stable and
about 35 per cent of its activity on standing at 4° for 2 weeks.

e enzyme appears to be relatively stable to heat. 5 minutes heating
° caused no inactivation, 50 per cent inactivation at 60°, and even at
nly 73 per cent inactivation. The sensitivity of the enzyme to acid
lkali was tested by adjusting aliquots of the enzyme solution to various
alues and permitting them to stand at room temperature for 1 hour.
pH was then adjusted to neutrality, the denatured proteins removed,
the supernatant fluids tested for activity in the usual way. The en-
e was essentially stable between pH 6.0 and 8.5, while pH 4.0 and 10.0
resulted in approximately 50 per cent inactivation.

ctivators and Inhibitors—The purified enzyme was found to lose activ-
on dialysis against water and phosphate, veronal, or maleate buffers
5 M) at a pH range of 6.0 to 7.7. In these cases there was an average
ne of 50 per cent in activity. This suggested a prosthetic group;
e the purified enzyme was ashed with nitric acid and examined spec-
copically[2] for metals. It was found that copper and magnesium were
ent at levels of 0.0376 and 0.0705 per cent respectively. However,
n these ions were added to the dialyzed or undialyzed enzymes over a
range of concentrations, no activation was observed. These experi-
ts were carried out in maleate buffer of pH 5.75 in order to minimize
tendency of the metal to form complexes with buffer components.
$^{+}$ and Mg^{++} in concentrations calculated to be 10-fold that present in
enzyme actually caused 20 to 25 per cent inhibition. Other metal ions
ed, *e.g.* Ca^{++}, Ba^{++}, and Zn^{++}, did not activate the enzyme, while
$^{++}$ and La^{+++} inactivated by precipitating the proteins.

hosphate ion was not required for the enzymatic hydrolysis of tri-

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Credit is gratefully extended to Dr. L. T. Steadman of the Atomic Energy Project
is University for the spectrochemical analysis of the protein for metals.

acetic acid and likewise possessed no reactivating effect on the di
enzyme. Diphosphopyridine nucleotide and adenosine triphosphat
similarily ineffectual.

Cyanide, iodoacetate, 8-hydroxyquinoline, and BAL in concentr
ranging from 5 × 10^{-4} to 1 × 10^{-3} M did not inhibit the enzyme. A
acetone and dimethylglyoxime (3 × 10^{-3} M) did not inhibit, while mal
oxalate, citrate, and benzoylacetone, each at 9 × 10^{-3} M, caused fr
to 45 per cent inhibition.

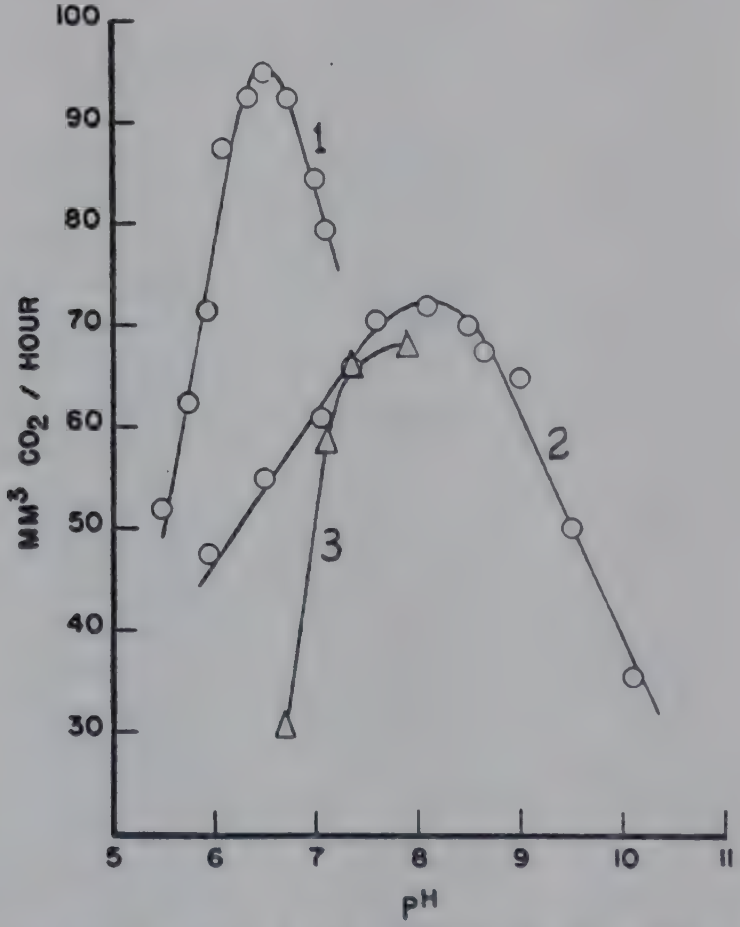
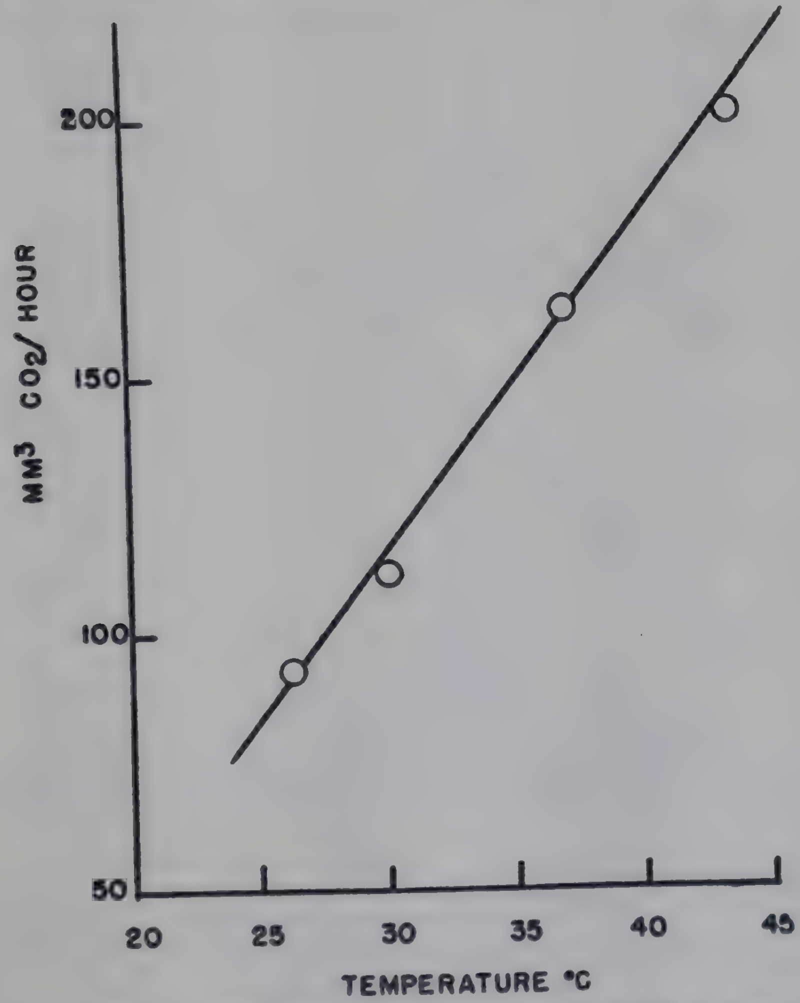


FIG. 2. Optimum pH. Curve 1, 0.025 M maleate buffer; Curve 2, 0.025 M
and Curve 3, 0.025 M phosphate; temperature 30°.

Optimum pH—The optimum pH range of the catalyzed reactio
studied in maleate and borate buffers. Colorimetric analysis of t
maining substrate was used to determine the rate of the reaction.
experimental conditions and results of these experiments are summ
in Fig. 2.

It may be noted that the optimum rate occurs at pH 6.5 in m
buffer, and at about pH 8.0 in phosphate and borate buffers (3.3 pe
mannitol was used to depress the pH in the borate solution when us
low pH 7.7).

perature Coefficient and Energy of Activation—Rate studies were con-l over the range of 26.3–43.7° in the Warburg apparatus with the CO_2-bicarbonate buffer. By means of the standard buffer concen- and gas phase it was calculated that the pH change over the tem-re range covered would be somewhat less than 0.3 of a unit. The um pH curves did not indicate that this deviation would be signifi- Fig. 3 illustrates the rate of the reaction plotted against tempera- From the straight line obtained over this range, the Q_{10} was calcu-



G. 3. Relation of reaction rate to temperature. 0.025 M bicarbonate buffer, .4.

l to be 1.60. When these data are substituted in the usual Arrhenius tion

$$E = \frac{2.303R \log K_2 - \log K_1}{\frac{1}{T_1} - \frac{1}{T_2}}$$

energy of activation of the catalyzed reaction was found to be 8670 ries per mole.

Michaelis-Menten Constant (K_m)—The relation of velocity of the r action to initial substrate concentration was studied. For this purpos colorimetric determination of the substrate was employed, since the low concentrations of substrate necessary for derivation of the Michaelis co stant were below the limits for satisfactory manometric assay. Velociti were determined from the initial straight line portions of the rate curve

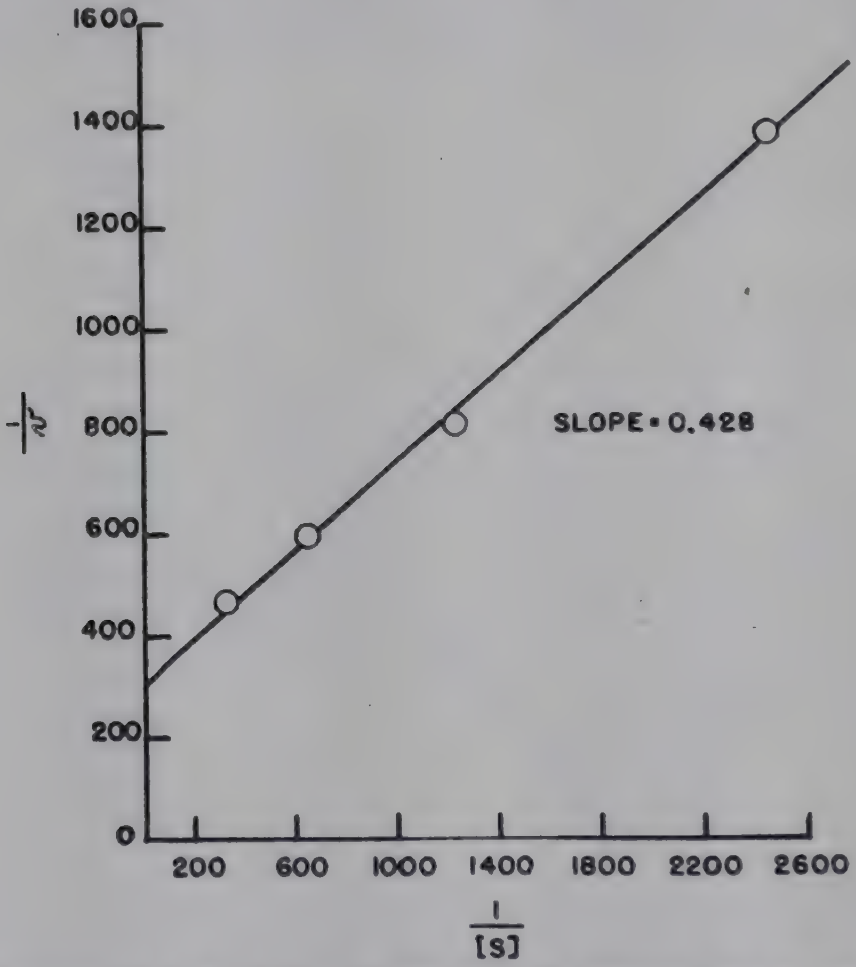


FIG. 4. Relation of $1/v$ to $1/[s]$. The velocity is expressed as moles of triace acid hydrolyzed per liter per hour, and $[s]$ is the initial substrate concentration moles per liter. 0.025 M maleate buffer, pH 6.5; temperature 30°.

and were expressed as moles of triacetic acid hydrolyzed per liter of re tion mixture per hour. Reciprocals of these velocities ($1/v$) are plotted ordinates in Fig. 4. Data for initial substrate concentrations are likew expressed as moles per liter, and reciprocals ($1/[s]$) are plotted as absciss A straight line relation was obtained by plotting $1/v$ against $1/[s]$. T Lineweaver-Burk (8) treatment of the data was employed; namely,

$$\frac{1}{v} = \frac{K_m}{V_{\text{max.}}(s)} + \frac{1}{V_{\text{max.}}}$$

The value of K_m was determined from the point of intercept $1/V_{\max.}$ and the slope $K_m/V_{\max.}$, and was found to be 0.00138 M.

Electrophoresis—Samples of the enzyme preparations from Stages III and IV were studied electrophoretically by Dr. Eric L. Alling to whom we are greatly indebted.

The descending boundaries are illustrated in Fig. 5. Two of the components were in close proximity and comprised some 84 per cent of the total protein. One of these, denoted by the arrow, increased markedly from the Stage III to Stage IV preparation, which corresponds to a doubling of the enzyme purity. Likewise, sampling of the components during electrophoresis indicated that the enzyme activity was associated with this component. On this basis our best preparation may be 25 per cent pure. On the other hand, it will be recalled that the enzyme is inactivated some 50 per cent by dialysis, and since dialysis is of course necessary prior to electrophoresis, it is not impossible that the component of slightly higher mobility represents modified or denatured enzyme. If this were true, our enzyme preparation would be considerably more pure.

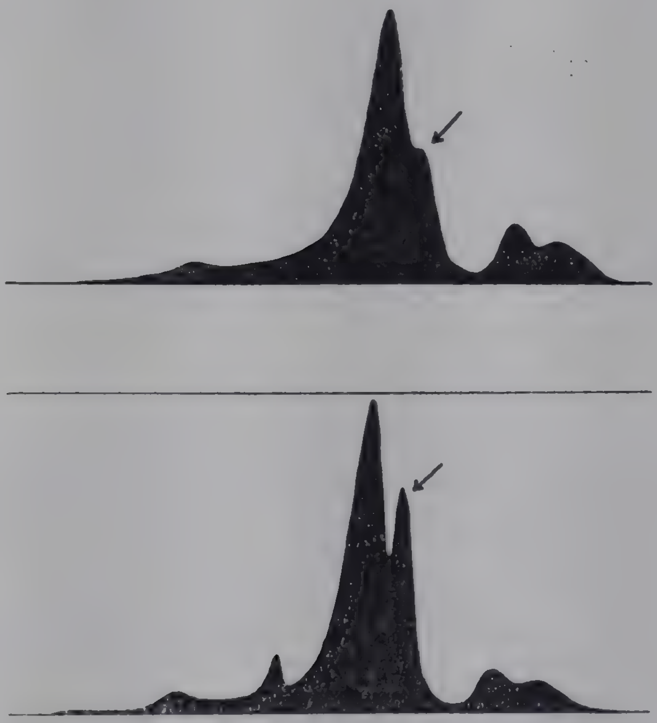


FIG. 5. Electrophoretic diagrams of purified enzyme. Longsworth scanning method. Veronal buffer, pH 8.5, ionic strength 0.1; temperature 1°; time 12,540 seconds; field strength 5.0 volts. Descending boundaries illustrated. Upper diagram, Stage III preparation; lower diagram, Stage IV preparation. Active component marked by arrow; its mobility $u = 2.4 \times 10^{-5}$ sq. cm. sec.$^{-1}$ volt^{-1}.

SUMMARY

An enzyme has been isolated from beef liver which catalyzes the re
tion

$$\underset{\text{Triacetic acid}}{CH_3COCH_2COCH_2COOH} \rightarrow \underset{\text{Acetoacetic acid}}{CH_3COCH_2COOH} + \underset{\text{Acetic acid}}{CH_3COOH}$$

It has been purified 100-fold by a combination of alcohol and acetone fr
tionations and heat denaturation steps. A number of related compou
have been tested for the specificity of this enzyme and of these only 3
diketohexanoic (triacetic acid) and 2,4-diketohexanoic acids were affect
The enzyme was not dependent on phosphate and a variety of cations
activity. The optimum pH was between 6.5 and 8.2, depending on
buffer used. The temperature coefficient of the catalyzed reaction
1.60, and its energy of activation calculated to be 8670 calories per m
Substrate velocity measurements indicated a typical enzyme-substr
relationship, and the Michaelis-Menten constant (K_m) was found to
0.00138 M.

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TRACER EXPERIMENTS ON THE MECHANISM OF URIC ACID DECOMPOSITION AND ACETIC ACID SYNTHESIS BY CLOSTRIDIUM ACIDI-URICI*

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(Received for publication, November 30, 1948)

Clostridium acidi-urici decomposes uric acid anaerobically (1) according to the equation

$$\text{Uric acid} + 5.5H_2O = 4NH_3 + 0.75CH_3COOH + 3.5CO_2 \qquad (1)$$

When a more reduced purine, such as xanthine, guanine, or hypoxanthine, is used as a substrate, the yield of carbon dioxide is smaller and that of acetic acid is larger. With hypoxanthine, for example, the equation is

$$\text{Hypoxanthine} + 6.5H_2O = 4NH_3 + 1.25CH_3COOH + 2.5CO_2 \qquad (2)$$

The relatively high yield of acetic acid from hypoxanthine is of considerable significance because a direct decomposition of the C_3 chain in 1 mole of hypoxanthine cannot give more than 1 mole of a C_2 compound. The observed yield of approximately 1.25 moles of acetic acid indicates that the formation of at least part of the acetic acid must involve a condensation either between two C_3 compounds, or between a C_3 and a C_1 compound, or between two C_1 compounds. The C_1 compound could be carbon dioxide or a derivative of the ureide carbons of the purine.

The possibility that carbon dioxide is used to form acetic acid was tested qualitatively several years ago by allowing uric acid or other purines to be fermented in the presence of $C^{11}O_2$ (2). It was found that carbon dioxide is incorporated into both the methyl and the carboxyl groups of acetic acid. Later experiments of the same type done with $C^{14}O_2$ provided quantitative data from which it was possible to conclude that a large part of the carboxyl carbon of acetic acid is derived directly from uric acid (3). The data also indicated that part of the methyl group may be derived from the same source, although other interpretations were not excluded.

There is considerable evidence to show that glycine is an intermediate in the decomposition of uric acid and other purines by anaerobic bacteria (1, 3). Although *Clostridium acidi-urici* does not form appreciable amounts of glycine as a fermentation product, the closely related species, *Clostridium cylindrosporum*, does so. Both species decompose glycine when it is added with a fermentable purine. As much as 1.55 moles of glycine may be decomposed per mole of uric acid when glycine is present in excess. In the absence of a purine, glycine is not appreciably decomposed. By the use of the Thunberg technique, glycine has been shown to function as a hydrogen donor for these bacteria. These facts indicate that glycine is formed in the fermentation of purines and is subsequently decomposed by serving as a reductant in an oxidation-reduction reaction with another purine derivative. Neither the mode of formation of glycine nor the products of its oxidation are known, though it has been shown that the carboxyl group of glycine is derived in part from carbon dioxide (3).

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In order to obtain further information concerning the mechanism of uric acid decomposition and acetic acid synthesis, we have carried out tracer experiments with C^{14}-labeled uric acid, glycine, and carbon dioxide. The results are reported in this paper along with some observations on the fermentability of some possible purine derivatives.

Materials and Methods

Strain 9a of *Clostridium acidi-urici* (4) was used in all experiments. The tracer experiments were done with growing cultures. A medium of the following composition in gm. per 100 ml. (Medium 1) was used unless otherwise stated: uric acid 0.168, glycine 0.75, Na_2CO_3 1.06, Difco yeast extract 0.3, K_2HPO_4 0.07, $MgSO_4$ 0.005, $CaSO_4$ 0.0006, FeSO 0.00025, and $Na_2S \cdot 9H_2O$ 0.015. The above medium without carbonate was neutralized to pH 7 with sodium hydroxide before autoclaving. After autoclaving the carbonate was added as a sterile solution and the pH was then adjusted to 7.8 with sterile 1 N hydrochloric acid. Cultures were incubated anaerobically at 37° until the uric acid was completely decomposed.

Relatively high concentrations of glycine and bicarbonate were used in the medium for two reasons. In the experiments with labeled uric acid the high initial concentrations of unlabeled glycine and bicarbonate resulted in a large isotopic dilution of the corresponding labeled compounds that might be formed during the fermentation. In this way the presence of more than one highly labeled carbon source was avoided. When labeled glycine or bicarbonate was used as a substrate, a relatively high initial concentration of the labeled compound prevented a considerable change in its specific activity during the fermentation. In all th tracer experiments with the exception of that with labeled bicarbonate th initial concentrations of uric acid, glycine, and bicarbonate were thos indicated in Medium 1. In the experiment with labeled bicarbonate it was considered desirable to minimize the isotopic dilution due to carbon dioxide produced from uric acid and glycine, and therefore the concentrations of the latter compounds were reduced to one-fourth of those used in other experiments.

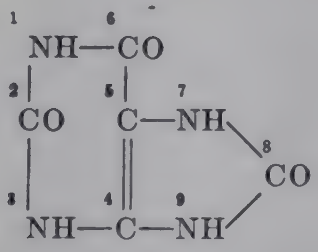
Cell suspensions were prepared by the method previously described (1).

Synthetic methyl-labeled and carboxyl-labeled glycine was used. Uric acid labeled in various positions was prepared biologically by injecting suitably labeled precursors into pigeons (5). Table I shows the distribution of C^{14} in the samples of uric acid used in the tracer experiments.

TABLE I

Distribution of C^{14} in Uric Acid Samples Used in Different Experiments

Experiment No.	Designation of labeled urate	Activity in carbon atom, counts per min. per mM					Total molar activity	Per cent of total activity in designated position
		Position 2	Position 8	Position 4	Position 5	Position 6	counts per min. per mM	
1	2, 8	237,000	237,000	1,300	5,080	1,030	481,410	98.5
2	4	0	0	3,950	450	100	4,500	87.5
3	5	6,530	6,530	6,460	22,000	820	42,340	52.0
4	6	470	470	28,000	4,840	100,800	134,580	75.0



For reference, the formula for uric acid is shown.

The data of Table I were obtained by the degradation methods used by Sonne, Buchanan, and Delluva (6, 7). These investigators showed that n uric acid derived from carboxyl-labeled acetate the C^{14} was equally distributed between the 2 ureide carbon atoms. In preparing Table I we have assumed that the same is true for the uric acid samples used in our experiments which were derived from labeled formate, glycine, or carbon dioxide.

Acetic acid was recovered from the fermented media by steam disillation and was degraded by decarboxylation of the barium salt at 550–600°. The resulting acetone was converted to iodoform, and the latter was oxidized to carbon dioxide and recovered as barium carbonate. The carbon dioxide in the fermented medium was precipitated as barium carbonate and then purified by dissolving the carbonate in acid, passing the evolved carbon dioxide through the gas phase, and reprecipitating the barium salt. The C^{14} content of the barium carbonate samples was estimated by means of a Geiger-Müller counter.

Results

Tracer Experiments with C^{14}-Labeled Uric Acid—The four kinds of labeled uric acid described in Table I were fermented in separate experiments by growing cultures in 100 ml. of Medium 1. Data on the distribution of C^{14} between the resulting carbon dioxide and acetic acid are given in Table II.

TABLE II

Fermentation of Radioactive Uric Acid in Presence of Excess Carbon Dioxide and Glycine

Experiment No.	Designation of labeled urate	Product		Specific activity	Total activity	Per cent of total substrate activity	Per cent recovery of C^{14}
			mM	*counts per min. per mM*	*counts per min. per mM*		
1	2, 8	CO_2	12.10	31,900	386,000	80	104
		HAc	1.03	112,500	116,000	24	
2	4	CO_2	10.05	400	4,020	89	92
		HAc	0.80	165	132	3	
3	5	CO_2	12.00	630	7,560	19	73
		HAc	0.92	25,000	23,000	54	
4	6	CO_2	12.06	3,550	42,800	32	33
		HAc	0.92	1,190	1,100	0.8	

Experiment 1 shows that the carbon of positions 2 and 8 went into both carbon dioxide and acetic acid. Approximately 80 per cent of the total activity appeared in the carbon dioxide and 24 per cent in the acetic acid. The high specific activity of the acetic acid in relation to that of the carbon dioxide proves that the labeled carbon in the acetic acid must have been derived more or less directly from the 2 and 8 positions rather than indirectly by way of carbon dioxide.

Experiment 2 shows that the carbon of position 4 went into carbon dioxide to a much greater extent than into acetic acid. The small amount of activity found in the acetate can be entirely accounted for by carbon originating from the 5 position of this sample of uric acid (Table I) and by carbon dioxide utilization. Therefore it must be concluded that there is little or no direct conversion of the carbon of position 4 into acetic acid.

Experiment 3 with 5-labeled uric acid was not quite as clear-cut as the

hers because the substrate was less specifically labeled. Table I shows
at almost half the total activity of this sample of uric acid was present
the 2, 4, and 8 positions. In spite of this difficulty considerable infor-
ation was obtained by the fermentation of 5-labeled urate. The entire
tivity of the carbon dioxide must have been derived from the 2, 4, and
positions. On the basis of the results obtained in Experiment 1, the
ntribution of the 2 and 8 positions to the carbon dioxide activity in Ex-
eriment 3 should have been 13,060 × (31,900/474,000) = 880 counts per
inute per mM. Based on the results of Experiment 2, the contribution
position 4 should have been 640 counts per minute per mM. The total
om both sources (1520 counts per minute per mM) is actually more than
wice the observed specific activity in the carbon dioxide of Experiment
(630 counts per minute per mM). Although the lack of quantitative
greement between the observed and expected specific activities leaves
uch to be desired, the data clearly indicate that very little if any of
he carbon from position 5 went into carbon dioxide. The carbon of posi-
ion 5 was used mainly for the synthesis of acetic acid. It is possible that
ome of the C^{14} unaccounted for was also present in glycine, although this
not certain.

In Experiment 4 the recovery of C^{14} from 6-labeled urate was lower than
ith uric acid labeled in other positions. This suggests that a large part
f the carbon from the 6 position was converted to glycine, although this
as not been demonstrated directly. Most of the C^{14} accounted for was
ound in carbon dioxide. Much of this must have originated from the
beled 4 position. On the basis of Experiment 2, the contribution of
osition 4 was estimated to have been 28,000 × (400/3950) = 2840 counts
er minute per mM. This leaves approximately 700 counts per minute
er mM that were derived from position 6. The small amount of activity
the acetic acid was probably derived from labeled carbon dioxide.

Tracer Experiments with Labeled Glycine or Carbon Dioxide—In the ex-
eriments with radioglycine the composition of the culture medium was
e same as in the above experiments except that unlabeled uric acid and
beled glycine were used. Since a large excess of glycine was present,
e change in its specific activity due to the production of inactive gly-
ine from uric acid was less than 10 per cent.

The data of Experiments 5 and 6 in Table III show that the methylene
arbon of glycine went exclusively into acetic acid, whereas the carboxyl
arbon went mainly to carbon dioxide. Most of the labeled acetate de-
ved from carboxyl-labeled glycine must have been formed from carbon
ioxide. However, a comparison of the ratios of the specific activities of
cetate and carbon dioxide in Experiments 7 and 4 suggests that a small
mount of acetic acid activity may have been derived directly from the
ycine carboxyl group.

As regards the utilization of carbon dioxide, Barker and Elsden (3) hav already shown that it is incorporated into acetic acid and glycine forme from uric acid by *Clostridium cylindrosporum*. During the course of thei experiments, the specific activity of the carbon dioxide changed so greatl that the quantitative contribution to the acetate carbon could not b estimated accurately. In our experiments with *Clostridium acidi-uric* a large excess of labeled carbon dioxide was added initially in order t minimize the isotope dilution effect. For the same reason the concentrations of uric acid and glycine were reduced to one-fourth of those used in the previous experiments. The significance of the experimental data given in Experiment 7 of Table III will be discussed below.

TABLE III

Fermentation of Uric Acid in Presence of Labeled Glycine or Labeled Carbon Dioxid

Experiment No.	Labeled substrate	Compound	mM per 100 ml.	Specific activit
				counts per min per mM
5	$\overset{*}{C}H_2NH_2COOH$	Glycine, initial	10.0	10,100
		CO_2, final	12.1	0
		HAc, "	1.24	3,300
6	$CH_2NH_2\overset{*}{C}OOH$	Glycine, initial	10.0	38,000
		CO_2, final	11.2	1,000
		HAc, "	1.30	800
7	$NaH\overset{*}{C}O_3$	CO_2, initial	$\sim$7.8	9,200
		" final	9.0	8,200
		HAc, "	0.33	3,000

TABLE IV

Distribution of C^{14} in Acetic Acid

Experiment No.	Labeled substrate	CH_3 group	COOH group
		counts per min. per mM	*counts per min. per mM*
1	2-, 8-urate	106,000	6,000
3	5-Urate	7,000	18,000
5	$\overset{*}{C}H_2NH_2COOH$	800	2,500
7	$NaH\overset{*}{C}O_3$	1,500	1,500

Distribution of C^{14} in Acetic Acid—Data on the distribution of the radioisotope in acetic acid derived from various labeled substrates are given in Table IV. In interpreting the data it must be borne in mind that the labeling of the uric acid used in Experiments 1 and 3 was not entirely specific.

The acetic acid derived from the 2,8-labeled urate (Experiment 1) was labeled mainly (95 per cent) in the methyl position. The small amount of activity in the carboxyl position must have been derived from the labeled 5 position or from carbon dioxide. On the basis of the results of Experiment 3, it can be estimated that the activity entering the carboxyl group of acetic acid from the labeled 5 position in Experiment 1 was approximately $5080 \times (18{,}000/22{,}000) = 4200$ counts per minute per mM. On the basis of Experiment 7, the carbon dioxide contribution to the carboxyl group should have been approximately $31{,}900 \times 0.5 \times (1500/8700) = 2700$ counts per minute per mM. The factor 0.5 is introduced because the average specific activity of the carbon dioxide during the fermentation was only about half its final specific activity. The sum of these two values (6900 counts per minute per mM) does not differ significantly from the observed value of 6000 counts per minute per mM.

The acetic acid derived from the 5-labeled urate (Experiment 3) contained more C^{14} in the carboxyl than in the methyl group. The carboxyl activity must have been derived almost entirely from position 5, since carbon from the 2, 4, 6, and 8 positions does not enter the carboxyl group directly and the contribution from the labeled carbon dioxide was very small ($\sim$200 counts per minute per mM). The activity in the methyl group must have been partially derived from the labeled 2 and 8 positions. The contribution from this source, based on the results of Experiment 1, should be $13{,}060 \times (106{,}000/474{,}000) = 2900$ counts per minute per mM. Since the contribution from carbon dioxide was very small, it may be concluded that approximately 4000 counts per minute per mM were derived directly from the 5 position.

The acetic acid formed in Experiment 5 with methylene-labeled glycine contained about 3 times as much activity in the carboxyl as in the methyl group. Since no activity was present in the carbon dioxide, all the observed activity in the acetic acid must have been derived directly from glycine.

The data of Experiment 7 indicate that the C^{14} was uniformly distributed in the acetic acid derived from labeled carbon dioxide. This result is not entirely in agreement with the data obtained by Barker and Elsden with *Clostridium cylindrosporum* (3). They found two-thirds of the activity in the methyl group and one-third in the carboxyl group. This difference between the two results could be due to a difference either in the organisms or in the experimental conditions.

Tests of Utilization of Possible Purine Derivatives—The only compounds known to be decomposed at an appreciable rate by *Clostridium acidi-urici* are uric acid, xanthine, guanine, hypoxanthine, and glycine. A number of other compounds, mostly purine derivatives and amino acids, were tested

previously either alone or in combination with uric acid and were f
not to be attacked. We have carried out tests of additional compo
that might conceivably occur as intermediates in purine decomposi
The tests were performed manometrically under anaerobic condi
with suspensions of cells grown on urate. An evolution of carbon di
in the presence of glycine and the compound being tested was take
an indication of utilization. Glycine was added to function as a hydr
donor so as to permit the recognition of compounds that can functi
hydrogen acceptors. Negative results were obtained with the follo
compounds: 5-amino-4-imidazolecarboxamide, 2,4,5-triamino-6-ox
rimidine, glutamate, aspartate, alanine, β-alanine, asparagine, ami
maric diamide, succinate, fumarate, malate, maleate, and pyru
The only compounds giving positive results were the four purines alr
known to be decomposed.

DISCUSSION

Since the first extensive studies were made on the fermentation of
acid and other purines by *Clostridium acidi-urici* and related bac
(1, 4), it has been evident that the process involves some unusual
chemical reactions. These studies showed that the mechanism of
acid decomposition by anaerobic bacteria must be quite different
that known to exist in aerobic organisms. Compounds such as alla
and urea, that are involved in the aerobic decomposition of uric acid
neither formed nor decomposed by the anaerobic bacteria.

The first definite evidence concerning the mechanism of the ferment
was the demonstration by the use of C^{11} that carbon dioxide is use
the synthesis of acetic acid (2). This qualitative observation led t
hypothesis that the anaerobic process involves a complete oxidati
the purine coupled with a reduction of carbon dioxide to acetic acid.
idea seemed very attractive because Wieringa (8) had recently disco
an anaerobic bacterium, *Clostridium aceticum*, that carried out an analo
process in which acetic acid was formed from hydrogen and carbo
oxide. However, further tracer experiments with C^{14} supplied info
tion that necessitated the abandonment of the simple carbon dioxid
duction hypothesis. It was found that the acetic acid, instead of
derived solely from carbon dioxide as this hypothesis predicts, is de
both from carbon dioxide and uric acid. The same is true of the gly
formed in small amounts during the fermentation of uric acid by
tridium cylindrosporum.

The data given in the present paper provide a much more com
picture of the rôle of the individual carbon atoms of uric acid, gly
and carbon dioxide in the fermentation process. Qualitatively,

nt that under the conditions used in our experiments acetic acid is ed from four sources, the 2 and 8 positions of uric acid, the 5 position c acid, the methylene carbon of glycine, and carbon dioxide. Car- lioxide is derived mainly from the 2, 4, and 8 positions of uric acid o a much lesser extent from the carboxyl carbon of glycine and the ition of uric acid. The origin of the glycine formed by *Clostridium lrosporum* has not as yet been thoroughly investigated, although are indirect indications that it may be derived from the 5 and 6 posi- in uric acid as well as from carbon dioxide.

ice all the tracer experiments were done under very similar conditions, esults of the different experiments are comparable, and it is possible fore to calculate the percentage of each carbon atom in the products ed from each carbon atom in the substrates. In general this per- ige is equal to the specific activity of the carbon atom in the product led by the specific activity of the carbon atom in the substrate. Of se, suitable corrections must be made for C^{14} entering the product from ond labeled source, if more than one is present, and for dilution of the e product by unlabeled material supplied in the medium.

ie method may be illustrated by calculating the percentage of the on dioxide derived from the 2 and 8 positions of the uric acid and the entage of the methyl carbon of acetic acid derived from the 5 position. ie former calculation it is assumed that the 2 and 8 positions contribute lly to the activity in the carbon dioxide. The activity derived from of the two positions will therefore be half of 31,900 or 15,950 counts ninute per mM. The desired percentage is therefore

$$\frac{15{,}950 \text{ counts per min. per mM}}{237{,}000 \text{ counts per min. per mM}} \times \frac{12.1 \text{ mM}}{3.11 \text{ mM}} \times 100 = 26$$

second factor, the ratio of the quantity of carbon dioxide in the medium e end of the experiment to that actually formed during the fermenta- corrects for the isotope dilution by added sodium bicarbonate. Since carbon dioxide production was not determined in this experiment, it calculated by multiplying the observed yield of acetic acid (1.03 mM) ie molar ratio in which carbon dioxide and acetic acid are formed (3.02). ratio was determined in a separate experiment carried out under ical conditions. The percentage of the carbon dioxide derived from the 2 and 8 positions is twice that derived from one position or 52. calculating the percentage of the methyl carbon of acetic acid derived the 5 position in uric acid, the observed specific activity of the methyl on (7000 counts per minute per mM) is first corrected by deducting ontribution from the 2 and 8 positions and from carbon dioxide by method already illustrated in a previous section. The corrected specific activity (4000 counts per minute per mM) divided by the spe activity of the 5 position (22,000 counts per minute per mM) gives a v of 18 per cent.

Table V summarizes all the available quantitative information on origin of the carbon atoms in the acetic acid and carbon dioxide for during the fermentation of uric acid in the presence of excess glycine carbon dioxide. The total for each carbon atom should add up to 100 cent. The observed totals are such as to indicate that the over-all er in the estimates are of the order of ±20 per cent.

Perhaps the most interesting and unexpected conclusion to be dr from Table V is that the 2 and 8 positions of uric acid contribute alm half of the methyl carbon of acetic acid. This is unexpected because

TABLE V

Origin of Carbon in Acetic Acid and Carbon Dioxide Formed in Uric A Glycine Fermentation

Experiment No.	Source	Per cent derived from each sou		
		HAc-CH_3	HAc-COOH	CO
1	Urate, 2 and 8 positions	45	2	52
2	" 4 position	0	0	44
3	" 5 "	18	82	0
4	" 6 "	0	0	3
5	Glycine, methylene group	8	25	0
6	" carboxyl group	~1*	~1*	7
7	Carbon dioxide	17	17	
Total		88	126	106

* The distribution of glycine carbon in the acetic acid was not determine 1:1 distribution is assumed.

2 and 8 carbon atoms are highly oxidized and because their conversio acetic acid requires a drastic rearrangement of the purine molecule the elimination of nitrogen and the formation of new carbon to car bonds. The other carbon atom mainly involved in the condensatio evidently that in the 5 position of uric acid, although carbon dioxide the methylene carbon of glycine also participate. The remainder of acetate methyl carbon comes from these same positions.

It is also noteworthy that the most reduced carbon atoms in the s strates, namely those in the 4 and 5 positions of uric acid and the methyl carbon of glycine, contribute heavily to the most oxidized carbon at in the products, namely those in carbon dioxide and the carboxyl grou acetic acid. For the glycine methylene group this result is the m surprising because tracer experiments with another anaerobic bacteri

ococcus glycinophilus, have shown that the methylene group of glycine
be converted preferentially into the methyl group of acetic acid (9).
was pointed out in the introduction that glycine functions as a power-
reductant when activated by *Clostridium acidi-urici*. On the basis
his observation it was thought that glycine might be oxidized com-
ely to carbon dioxide. It is now apparent that this does not occur.
y the carboxyl group is converted to carbon dioxide. The methylene
on evidently takes part in a condensation with one or more of the
on atoms from uric acid that leads to the formation of acetic acid.
ess than one-fifth of the acetic acid is derived from carbon dioxide.
s obvious therefore that carbon dioxide reduction is not as important
n a quantitative point of view as was previously supposed (2).

he general conclusion to be drawn from the tracer experiments is that
fermentation of uric acid is a very complex process which involves
ariety of condensation and oxidation-reduction reactions. The fur-
r elucidation of these reactions can probably best be achieved by en-
natic methods.

he fermentation tests reported in the experimental section are of
rest because they eliminate several compounds from consideration as
sible intermediates in the breakdown of purines. The compound
t seemed most promising in this respect was 5-amino-4-imidazolecar-
amide. This compound, which is similar to xanthine except that the
on atom in the 2 position is missing, is believed to be formed from
ine by *Escherichia coli* (10). But since it is not attacked by cell
ensions of *Clostridium acidi-urici*, it is unlikely to be an intermediate
he fermentation of purines.

SUMMARY

he fermentation of purines by *Clostridium acidi-urici* has been investi-
ed by the use of C^{14}-labeled uric acid, glycine, and carbon dioxide.
results show that the methyl carbon of the acetic acid formed when
acid is fermented in the presence of excess glycine and bicarbonate
erived from the 2, 5, and 8 positions of uric acid, the methylene carbon
lycine, and carbon dioxide. The acetic acid carboxyl carbon is derived
nly from the latter two sources and from the 5 position of uric acid.
carbon dioxide formed in the fermentation comes mostly from the
, and 8 positions of uric acid, and to a lesser extent from the 6 position,
from the carboxyl group of glycine. Several compounds including
ino-4-imidazolecarboxamide have been eliminated as likely inter-
iates in the purine fermentation.

he authors are greatly indebted to the following persons who gener-
y supplied synthetic compounds used in this investigation: Dr. Wil-

liam Shive (5-amino-4-imidazolecarboxamide), Dr. H. K. Mitchell (amin
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HE EFFECT OF VARIOUS COMPOUNDS ON UREA FORMATION BY RAT LIVER SLICES

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In studies on the synthesis of urea *in vitro*, the nitrogen is usually supplied s NH_4Cl or as $(NH_4)_2SO_4$ (1–3). It has been suggested that the mechaism by which such nitrogen enters the cycle and forms urea is somewhat lifferent from that utilized by nitrogen supplied in the form of glutamine or mino acids. Leuthardt and Glasson (2) believe that ammonia from inorganic salts enters the cycle directly but that ammonia from amino acids may e synthesized into glutamine, which then transfers it to ornithine. Once itrulline is formed, the second molecule of ammonia is added and the reaction is catalyzed by adenosine triphosphate and glutamic acid, as shown by Cohen and Hayano (3). The source of the carbon dioxide which, along with he nitrogen, is added to ornithine to form citrulline can be CO_2 supplied y sodium bicarbonate, as recently proved by Grisolia and Cohen (4) but reviously indicated by Leuthardt and Glasson (2), who showed that urea ormation from ammonium salts was greatly decreased when liver slices vere incubated in phosphate instead of bicarbonate medium. The latter lso extended the original observations of Krebs (1) by demonstrating that he addition of pyruvate or oxalacetate increased the urea production in the bsence of bicarbonate, presumably by supplying CO_2. The following is a eport of experiments with liver slices, comparing urea production from an mmonium salt, glutamine, and an amino acid under varying conditions.

EXPERIMENTAL

The livers of white rats weighing 200 to 250 gm. were used. The animals vere usually fasted for 24 hours before use. The liver slices, 250 to 300 mg. (wet weight), were placed in 50 ml. Erlenmeyer flasks which contained 4.0 nl. of either Krebs' phosphate or bicarbonate solution. The flasks were incubated at 37° for $2\frac{1}{2}$ to 3 hours. A gas mixture of 95 per cent O_2 and 5 per ent CO_2 was used with the bicarbonate solution, and 100 per cent O_2 with the phosphate solution. At the end of the experiment, 1.9 ml. of H_2O and .1 ml. of $\frac{2}{3}$ N H_2SO_4 were added to each flask; the liquid was poured off the slices and placed in a boiling water bath for 5 minutes. The precipitated protein was centrifuged and aliquots were taken for the estimation of urea by

Barker's method (5), and for the estimation of ammonia by direct nessleri tion.

A typical experiment on the effect of added ornithine on the amount urea produced from $(NH_4)_2SO_4$, L-glutamine, and L-alanine is shown Table I. In Table I, and in all others, the amount of urea formed by t slices alone has been subtracted. The concentration of each was such as insure an excess and the maximum urea production under the conditions the experiment. Under these conditions the addition of ornithine increas the amount of urea formed from $(NH_4)_2SO_4$, but has little effect on th formed from the other two compounds. This fact suggests that the or thine concentration becomes a limiting factor for $(NH_4)_2SO_4$ before it d for glutamine or alanine. Thus, Krebs' observation that certain amino aci added with ammonium salts to slices cause less urea to be formed than ammonium salts alone may be explained when it is assumed that the ami acids utilize the available ornithine at the expense of the $(NH_4)_2SO_4$. N comment has been made on this inhibition and it has been largely ove looked. Recently, Leuthardt, Fahrländer, and Nielsen (6) obtained t same effect with guinea pig liver. Alanine, glycine, serine, methionin cystine, and lysine inhibit urea production from NH_4Cl in the presence pyruvate and ornithine. Histidine and proline do not. They found th the inhibition was much less in rat than in guinea pig liver. They studi the effect of methionine and cysteine in detail on liver suspensions and ca to the conclusion that these two amino acids bound magnesium, for the ad tion of the latter overcame the inhibition. They did not determine the fect of added magnesium in rat liver slices, but showed that, in these, t formation of citrulline from ornithine was inhibited by methionine. have been able to extend and confirm these results in part. To the list inactive amino acids, valine, leucine, and aspartic and glutamic acids can added, while phenylalanine acts as an inhibitor. D-Methionine is as acti

TABLE I

Effect of DL-*Ornithine Hydrochloride on Urea Production*

0.3×10^{-2} M DL-ornithine hydrochloride; 0.76×10^{-2} M $(NH_4)_2SO_4$; 1.36×1 M L-glutamine; and 2.20×10^{-2} M L-alanine.

Substrate	Urea N formed
	γ
$(NH_4)_2SO_4$	149
" + ornithine	310
Glutamine	324
" + ornithine	318
Alanine	193
" + ornithine	204

he L isomer, despite the fact that it produces little urea. However, the ults under our conditions with rat liver slices differ somewhat from those Leuthardt *et al.* (6). Glycine is more active than methionine, and

TABLE II

Effect of Various Amino Acids on Urea Production from $(NH_4)_2SO_4$

Except in Section *e*, where the effect of different concentrations of glycine and thionine are shown, the concentration of amino acids was 1.33×10^{-2} M.

ction No.	Compounds added	Urea N formed	Inhibition
		γ	*per cent*
a	$(NH_4)_2SO_4$	283	
	“ + DL-methionine	107	62
	“ + L-phenylalanine	118	58
	“ + DL-phenylalanine	110	61
	“ + glycine	33	85
b	“	216	
	“ + DL-leucine	205	0
	“ + L-valine	230	0
	“ + L-histidine	224	0
	“ + L-cysteine	146	28
	“ + glycine	48	78
c	“	188	
	“ + L-aspartic acid	214	0
	“ + DL-serine	67	64
	“ + glycine	50	74
d	“	306	
	“ + DL-lysine	164	46
	“ + L-glutamic acid	298	0
	“ + L-alanine	268*	12
	“ + glycine	46	82
e	“	294	
	“ $+ 0.67 \times 10^{-2}$ M L-methionine	192	35
	“ $+ 1.33 \times 10^{-2}$ “ “	143	51
	“ $+ 0.67 \times 10^{-2}$ “ DL-methionine	188	36
	“ $+ 1.33 \times 10^{-2}$ “ “	141	52
	“ $+ 0.34 \times 10^{-2}$ “ glycine	197	33
	“ $+ 0.67 \times 10^{-2}$ “ “	173	41
	“ $+ 1.33 \times 10^{-2}$ “ “	124	58
	“ $+ 1.33 \times 10^{-2}$ “ DL-proline	286	0

* NH_3-N determinations show that alanine, not $(NH_4)_2SO_4$, was the source of ost of the urea. The apparent inhibition is therefore too small.

hibits urea production as much as 80 per cent; this can be completely vercome by the presence of sufficient ornithine. The addition of excess agnesium has apparently no effect. The effect of various amino acids is hown in Table II, which also shows that the amount of urea formed from

$(NH_4)_2SO_4$ varies considerably from animal to animal. These amin
added to liver slices, produce some urea. When both $(NH_4)_2SO_4$
amino acid are present, it is not possible to determine how much ea
tributes to the urea formation. The inhibitions are therefore calcul
the assumption that no urea has originated from the amino acid, mak
values lower than they actually are. This is particularly true in the
alanine, which produces more urea by itself than any of the other amin
Glycine does not inhibit arginase under the conditions of the experime
in fact, Hunter and Downs (7) have shown that it has no effect on the
fied arginase preparation. The glycine inhibition occurs both in ph
and in bicarbonate medium, and in neither does the addition of pyruva
come it. Glycine does not affect urea production from glutamine or
It seems probable, therefore, that glycine, and possibly other active
acids, inhibits urea formation from $(NH_4)_2SO_4$ by preferentially utiliz

TABLE III

Effect of Various N-Substituted Glycines (1.88×10^{-2} M) on Production of U
$(NH_4)_2SO_4$

Compounds added	Urea N formed	Inhibit
	γ	per c
$(NH_4)_2SO_4$	218	
" + glycine	25	88
" + sarcosine	42	81
" + dimethylglycine	86	60
" + ethylglycine	133	39
" + hippuric acid	96	57

available ornithine and reducing the concentration below the level nee
the $(NH_4)_2SO_4$. The fact that added ornithine overcomes the inh
supports this view. Glycine causes the same percentage inhibitio
smaller amounts of $(NH_4)_2SO_4$. Larger amounts of $(NH_4)_2SO_4$ are

Table III shows the effect of N-substituted glycines. Sarcosine is
as effective as glycine, dimethylglycine is less effective, and ethylglyc
least. This is the order in which they are attacked by the sarcosine
(8), so that probably substitution on the nitrogen decreases the inh
and most of the effect caused by these compounds depends upon the a
of free glycine produced. By analogy the hippuric acid inhibition m
due in part at least to the free glycine formed by hippuricase. Benz
equimolar concentrations has no effect on urea production.

It has been shown (9) that caffeine inhibits urea formation from (
SO_4 and that added ornithine overcomes the inhibition. Caffeine ha
effect on urea formed from glutamine and alanine. It thus apparent

e amino acids. 2,4-Dinitrophenol is another drug which acts dif-lly on urea production from organic and inorganic nitrogen sources. wn in Table IV, concentrations of dinitrophenol of 0.7 and 1.4 × markedly inhibit urea formation from $(NH_4)_2SO_4$ in the presence of ornithine, in liver slices from rats fasted 24 hours, but they have much ect when alanine is used and the glutamine reaction is not inhibited. me is true if ornithine is not added. Trinitrophenol is inactive, even nes the concentration.

e (1) and Leuthardt and Glasson (2) have shown that, in liver slices rom a fasted animal, urea formation is increased by the addition of te, and the latter have shown that oxalacetate is even more effective.

TABLE IV

f 2,4-Dinitrophenol on Urea Formation from $(NH_4)_2SO_4$, L-Glutamine, and L-Alanine

oncentrations were the same as in Table I.

Substrate	Dinitrophenol	Urea N formed	Inhibition
		γ	*per cent*
O_4 + ornithine		460	
+ "	0.7 × 10^{-4} M	250	46
+ "	1.4 × 10^{-4} "	55	88
ne + "		432	
+ "	0.7 × 10^{-4} "	448	0
+ "	1.4 × 10^{-4} "	398	8
+ "		367	
+ "	0.7 × 10^{-4} "	302	18
+ "	1.4 × 10^{-4} "	257	30

te and citrate also have some effect. In our experiments, both e and oxalacetate increase the amount of urea formed by liver om fasted animals from $(NH_4)_2SO_4$ with added ornithine in bi-te medium, but they do not affect the urea formed from alanine or ne.

rophenol causes less inhibition in liver slices from well fed than from nimals. Pyruvate, added to the liver slices of fasted rats, almost ely overcomes this inhibition. Oxalacetate, however, does not. hown in Table V. Concentrations higher than those given have no effect. Lactate is as active as pyruvate, probably because it is oxidized to it, but succinate, fumarate, citrate, acetate, and glucose vercome the inhibition. The addition of glutamic acid and adeno-phosphate together decreases the inhibition to a small extent only), which indicates that dinitrophenol acts primarily by inhibiting ation of citrulline from ornithine.

Since oxalacetate does not overcome the dinitrophenol inhibition, it unlikely that pyruvate is acting simply as a carbon dioxide source. Fu ther evidence against this possibility is the fact that pyruvate is witho effect when the reaction is run in phosphate medium. It appears, ther fore, that dinitrophenol is preventing the addition of ammonia and C to ornithine. Pyruvate circumvents this effect because it reacts with am monia to form alanine, as shown by Neber (10), who used rat liver slice the urea production from alanine thus formed would be only slight inhibited by dinitrophenol.

TABLE V

Effect of Pyruvate, Oxalacetate, and Glutamic Acid Plus Adenosine Triphosphate Inhibition by 0.7 × 10^{-4} M Dinitrophenol of Urea Formation from $(NH_4)_2SO_4$ and DL-*Ornithine HCl*

$(NH_4)_2SO_4$ and ornithine + additions	Urea N formed	Inhibition
	γ	*per cent*
None	224	
+ dinitrophenol	49	78
+ 0.45 × 10^{-2} M pyruvate	398	
+ 0.45 × 10^{-2} " " + dinitrophenol	132	67
+ 0.9 × 10^{-2} M pyruvate	420	
+ 0.9 × 10^{-2} " " + dinitrophenol	294	30
+ 0.38 × 10^{-2} M oxalacetate	298	
+ 0.38 × 10^{-2} " " + dinitrophenol	85	72
+ 0.76 × 10^{-2} " "	324	
+ 0.76 × 10^{-2} " " + dinitrophenol	115	65
None	406	
+ dinitrophenol	107	74
+ 0.9 × 10^{-2} M pyruvate	420	
+ 0.9 × 10^{-2} " " + dinitrophenol	366	13
+ 0.7 × 10^{-2} M glutamic acid + 2 × 10^{-3} M adenosine triphosphate	455	
None + 0.7 × 10^{-2} M glutamic acid + 2 × 10^{-3} M adenosine triphosphate + dinitrophenol	225	51

DISCUSSION

Urea production from ammonium salts requires higher ornithine conce trations than urea production from glutamine or alanine. Certain ami acids inhibit urea production from ammonium salts but not from glutami and alanine. The conclusion from these facts is that the nitrogen in glut mine and alanine has a greater affinity for ornithine than the inorganic nitr gen. This could hardly be so if glutamine and alanine were directly aminated. The implication is either that these two compounds and oth amino acids as well have an affinity for ornithine higher than that of a onium ions, and thus combine with it before or synchronously with the eamination process, or that they form carbamino compounds, as suggested Leuthardt and Glasson (2), so that carbon dioxide is transferred with the nmonia to ornithine. Dinitrophenol in low concentrations blocks the mbination of ornithine with ammonium salts, but the latter can be synesized to alanine when pyruvate is added and can give rise to urea by this eans.

SUMMARY

1. The addition of ornithine to liver slices from rats fasted 24 hours ineases the urea production from $(NH_4)_2SO_4$ but has little effect on that from alanine or L-glutamine.

2. Certain amino acids, particularly glycine, inhibit urea production from $H_4)_2SO_4$. This inhibition can be overcome by adding ornithine but not agnesium salts. Glycine has no effect on urea production from alanine glutamine. In these respects the caffeine inhibition resembles the glycine hibition.

3. In low concentrations 2,4-dinitrophenol inhibits urea production from $H_4)_2SO_4$, but has much less effect on that from alanine and no effect on at from glutamine. The inhibition occurs in the presence of ornithine but n be partially overcome by the addition of pyruvate, probably enabling e ammonia to be synthesized to alanine, which then acts as the source of ea.

4. The conclusion is drawn that the mechanism of the addition of ammonia ornithine differs when inorganic and organic nitrogen sources are used.

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THE CREATINE CONTENT OF THE LIVER IN THE MUSCULAR DYSTROPHY OF VITAMIN E DEFICIENCY

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Creatinuria is one of the results of vitamin E deficiency. This was first emonstrated by Morgulis and Spencer (1) on rabbits. The effectiveness f α-tocopherol in reducing the excretion of creatine to normal levels was hown by Mackenzie and McCollum (2) and has been verified by many thers. The creatine lost from muscle tissue during dystrophy (3) unoubtedly accounts for the creatinuria, and the administration of α-ocopherol prevents this loss (4). Biopsy experiments on dystrophic rabits (5) indicated that the restoration of normal muscle creatine content, fter the administration of tocopherol phosphate by vein, was delayed ome hours as compared with relatively prompt reduction of the high xygen consumption. This latter dropped in about 4 hours, whereas the lready low creatine content was initially still further reduced. A sugested explanation for these changes was that the loss of creatine from uscle continued, while a previously accelerated process of synthesis had lready been depressed.

There is no proof that the synthesis of creatine is accelerated in dystrophic nimals, but the high rate of excretion would quickly exhaust the existing tores unless these were being replenished at a greater than normal rate.[1]

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[1] No direct evidence is available on this question. From existing data, only an pproximate calculation can be made. If the creatine content of normal rabbit uscle is 0.5 per cent, the total body creatine of an animal weighing 1 kilo would be 00 × 0.4 × 5 mg., or 2000 mg. ((6) p. 494). The excess creatine excretion of a ystrophic rabbit ((2) p. 354) over a period of 27 days was approximately this same mount. If the dystrophic muscles still contained 1280 mg. (800 × 0.4 × 4 mg.) f creatine ((7) p. 304), this amount had to be manufactured during the 27 days, in ddition to that which continued to be excreted as creatinine rather constantly hroughout the period. If urinary creatinine has its origin in the phosphocreatine f muscle (6), the creatinine excretion of dystrophic animals should be reduced. ince it is not, the prior transformation of creatine to phosphocreatine may be cking or incomplete in these animals. In dystrophic muscle the decline of phoshocreatine seems to be greater than that of creatine (8). Coupled phosphorylaon of creatine has, in fact, been shown to be greatly diminished in homogenates of ystrophic muscles from guinea pigs and hamsters (9), but this could not be demon-

The liver is apparently the principal site of final creatine synthesis If the production of creatine is accelerated in vitamin E deficiency, a hi concentration of it might be expected in the liver, even though this o may not store it extensively. In any case, the active participation o liver in the fluctuations of creatine can hardly be excluded.

It seemed important, therefore, to examine into the rôle of the liv the alterations of creatine metabolism found in vitamin E deficiency. beginning was made by determining the creatine (and creatinine) con of liver tissue from normal rabbits and rats and from these ani after various periods of maintenance on vitamin E-deficient diets. layed and immediate effects of the administration of α-tocopherol ac or phosphate were also studied.

EXPERIMENTAL

The liver contains large amounts of chromogens other than creati which respond to the Jaffe test. Since absolute and not relative value creatine were desired, the specific enzymatic method of Miller, Allin and Baker (11) was chosen, even though it is cumbersome and has abandoned by some (12–14).

Lloyd's reagent was found to be unsatisfactory for the separatio creatinine from other chromogens found in autoclaved liver; it was specific and variable in its behavior. In addition to removing creati it adsorbed other Jaffe-positive chromogens and additional colored pounds. Shaking 25 mg. of the reagent with 6 ml. samples of liver fil for 2 minutes removed as much as 40 per cent of the color. Incuba the filtrate with bacteria removed no color. Furthermore, the amou chromogen removed by Lloyd's reagent was variable, from a fractio several times the amount of creatinine removed from the same filtrat bacterial digestion. After longer periods of shaking, some of the color chromogen originally adsorbed by Lloyd's reagent returned to the solu This is not a new observation.

The suspensions of *Corynebacterium creatinovorans* (NC)[2] were prep and assayed according to the recommended procedure (11).

Minor changes were made in the protein precipitation preceding c inine estimation, and in the order of color development. The pro were precipitated by the acid cadmium reagent as used in the ori method of Fujita and Iwatake (15). A simplification was introduce

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strated with rabbit muscle. A study of creatine-creatinine excretion as re to muscle creatine and phosphocreatine in dystrophic animals is greatly neede

[2] Obtained from the American Type Culture Collection.

[3] The help of Dr. J. R. Porter and the Department of Bacteriology in cult the bacteria is gratefully acknowledged.

ing at one time all the sodium hydroxide required to precipitate the mium. This had no effect upon the completeness of protein precipi-on or on the final creatinine values. In the modified procedure, 8 ml. cid cadmium reagent were added to the tissue extract, followed by 1.5 of 1.1 N sodium hydroxide. The mixture was allowed to stand 5 utes before filtering; the filtrate was used directly for color development ncubation. With large samples of liver, the glycogen extracted gave idy solutions which were not clarified by the cadmium treatment. A ett-Summerson photoelectric colorimeter was used with filter No. 54.

ince in liver tissue the true creatine and creatinine usually constitute y 5 to 25 per cent of the total chromogen, the difference between the omogen content before and after bacterial digestion is very small.

Vith liver filtrates the intensity of the color with picrate was found to rease for at least 45 minutes, whereas with pure solutions of creatinine ximum color development is attained in 12 minutes. In order to have or development simultaneous on each sample of original and incubated rates and on a blank, the addition of alkaline picrate was properly timed reading after exactly 12 minutes. Notwithstanding these precautions, occasional liver filtrate contained more chromogen after digestion than ore, possibly as a result of bacterial synthesis. When this happened, results were discarded.

abbits weighing 800 to 1000 gm. were maintained on the same vitamin leficient diet as was used heretofore (7), except that the carbohydrate s sometimes glucose only. After about 1 month on this diet, or less, en an animal was unable to rise after being placed on its side, it was led by stunning; the liver and gastrocnemii, or portions of them, were ighed wet, minced, and analyzed for creatine and creatinine. The erage weights of liver used for single creatine and creatinine estimations re about 2 and 8 gm. respectively, of muscle tissue about 0.5 and 2 gm. pectively. Control rabbits were fed the same vitamin E-deficient diet, h a supplement of 10 to 15 mg. per week of *dl*-α-tocopherol acetate[4] en by mouth in olive oil.

he results of these analyses are given in Table I. In agreement with observations of many workers not using the specific enzymatic method, muscle of vitamin E-deficient animals contained much less creatine n is normally present. The amount of true creatine in the liver of mal rabbits is in the range of that reported for rats and dogs (16). No or figures seem to be available for the rabbit. Liver tissue from vitamin leficient rabbits showed an appreciable accumulation of creatine, 2 3 times the amount in the livers of control animals. This increase was

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dl-α-Tocopherol acetate and α-tocopherol phosphate disodium salt were gen-
usly supplied by Hoffman-La Roche, Inc.

not in total chromogen but only in creatine. The characterization of
degree of severity of dystrophy is rather uncertain at best; this lack of
adequate criteria probably accounts for the great variability in the li
creatine figures. The creatinine content of both liver and muscle wa
reasonable agreement with the figures for rats (16), and was so small t
its determination was not continued.

A more direct method of studying the effect of vitamin E on creat
production in the liver was obviously desirable and biopsy experime
were therefore undertaken. Vitamin E-deficient rabbits were used
biopsy experiments when dystrophy was well developed. Control anim
received the same diet plus 5 mg. of *dl*-α-tocopherol acetate in olive
daily.

Table I

True Creatine and Creatinine in Muscle and Liver of Rabbits

The results are given in mg. per 100 gm. of tissue.

Rabbit	Creatine		Creatinine	
	Muscle	Liver	Muscle	Liv
Vitamin E-deficient	245	42.6	0.6	0
" "	107	11.9	0.9	0
" "	292	37.6		0
" "	128	34.3	0.8	0.
" "	148	46.8	2.0	0.
" "	139	15.0	0.8	0.
Control	426	10.3	3.7	
"		5.6*		
"		3.8*		

* Average of duplicates from later biopsy experiments.

Anesthesia for the duration of the operation was provided by divi
doses of sodium amytal given intraperitoneally. Amytal appeared to h
no effect on liver creatine. Severe hemorrhage was avoided by remov
liver samples with electrosurgery,[5] and by the use of thrombin[6] solut
After removal of an initial sample through a mid-line incision, 10 to 25
of disodium-α-tocopherol phosphate in distilled water were injected
the femoral vein. Further liver samples were removed at intervals of
4 hours. The incision was kept closed and the animal was covered
kept warm. Each liver specimen of 0.5 to 2 gm. was immediately min
divided into two portions, weighed, and autoclaved with acid for crea
analysis by the enzymatic method. Large stock animals were easily m

[5] Apparatus kindly loaned by Dr. W. R. Ingram, Department of Anatomy.

[6] Generously supplied by Dr. H. P. Smith, Department of Pathology.

ined in excellent condition under anesthesia for 12 hours, during which many as nine samples of liver could be removed. Dystrophic rabbits, wever, lived no longer than 4 hours after the injection of tocopherol osphate. Their smaller size and weakened condition were probably sponsible; a contributory factor may have been too large doses of tocopherol phosphate.

Table II shows the effect of the intravenous administration of α-tocopherol phosphate upon liver creatine. The changes were not significant, cept in one animal, whose high value of 65 mg. per cent had dropped to out 40 mg. per cent 3 hours after the injection of 10 mg. of the phosphate.

TABLE II

Effect of Tocopherol Phosphate on Liver Creatine of Rabbits

The results are given in mg. of creatine per 100 gm. of tissue; duplicate analyses.

Rabbit	Tocopherol phosphate injected	Initial creatine	Creatine after injection				
			½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.
	mg.						
Vitamin E-deficient	25	15.4	22.7		24.6		21.8
		18.9	28.8		31.2		20.0
" "	15	31.5		27.9			
		20.9		27.5			
" "	15	41.4		42.2	34.1		
		34.1		32.9	37.7		
" "	10	65.5				36.6	
		64.0				40.7	
						43.5	
Control	25	3.5		4.1			
		4.0		2.5			
"	15	6.2		2.6	3.0		
		4.9			2.9		

another, a large dose (25 mg.) of tocopherol phosphate was followed by increased creatine level during the first 2 hours, followed by a decline vard the initial value. In this case the initial creatine level was low, haps implying a less severe dystrophic condition. Unequal distribution creatine between the different lobes is another possible variable which ıld not be controlled. Apparently any effects of tocopherol injection nnot be expected in less than 3 or 4 hours, and unless the deficient anils can be made to survive for a longer time, the approach by the techue of biopsy is not promising. In control animals, the effect of the ections was indifferent and not significant.

Vith some hesitation, attempts were made to demonstrate the effects of ocopherol acetate on the creatine content of the livers from vitamin

E-deficient rats. Some of the animals had been on the deficient die
more than a year and were almost completely paralyzed. Other you
animals showed only slight disability in the use of the hind legs, and
others showed few if any external symptoms. Doses of α-tocop
acetate, 10 mg. per day, in divided doses, for 3 days preceding liver ana
produced no apparent change in their condition, as was to be expected
no significant change in liver creatine. The figures for control ani
were similar to those found for control rabbits, 3 to 7 mg. per 100 gr
tissue. The highest value in the deficient rats was 9.8 mg. per 100 g

DISCUSSION

The negative results with rats are not surprising in view of the dissim
ities in the responses of rabbits and rats to vitamin E deficiency.
muscle function, the need of the maturing rabbit for tocopherol is cri
that of the rat is not, once the nursling stage has been passed. Differe
in cellular metabolic processes must be the determinants in these variat
The guinea pig is subject to dystrophy, like the rabbit, and its liver
mogenates methylate guanidoacetic acid at double the rate found fo
liver slices (14).

The high content of creatine in the livers of vitamin E-deficient rab
is in keeping with the notion that the rapid turnover of creatine du
dystrophy requires its synthesis at an abnormally rapid rate. Perhaps
administration of tocopherol phosphate slows up the accelerated proce
creatine synthesis sooner than it restores the capacity of the muscle
conserve creatine by phosphorylation. This order of events would acc
for the preliminary decrease in the already diminished muscle crea
when α-tocopherol is given to dystrophic rabbits (5).

A satisfactory solution of the problem will require a study of crea
synthesis in liver slices from dystrophic animals, and a complete bal
sheet of liver, muscle, blood, and urinary creatine. Such studies wi
greatly facilitated by less circumstantial and more precise method
determining creatine in tissues as complex as liver, and in the blood.

SUMMARY

Creatine and creatinine were determined in the liver and skeletal mu
of vitamin E-deficient rabbits by the enzymatic method.

The concentration of creatine in the liver was much higher in dystro
than in control animals; in both cases the creatinine content was small
without significant differences.

In biopsy experiments, the level of liver creatine of vitamin E-defic
rabbits was not lowered within 4 hours after injection of α-tocoph
phosphate.

e injection of tocopherol phosphate had no influence on the liver
ne of control animals. The possibility is discussed briefly that lack
amin E in the rabbit involves an increased rate of synthesis of creatine
e liver and a decrease in the rate of its phosphorylation in muscle.

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THE OCCURRENCE AND PHYSIOLOGICAL BEHAVIOR OF TWO METAPHOSPHATE FRACTIONS IN YEAST

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The synthesis of basophilic substances observed by Jeener and Brachet
in yeast cells has been shown to be due chiefly to the formation of meta-
osphate (2–4). The presence of metaphosphate in yeast had already been
served by Kossel (5) in 1893, by Macfarlane (6) in 1936, and recently in
pergillus niger by Mann (7). It has also been shown (2, 3) that the meta-
omatic material which can be demonstrated in yeast by histochemical
ining reactions is metaphosphate. In fact, hexametaphosphate as well as
metaphosphate extracted from yeast gives the metachromatic reaction
solution. This reaction is very sensitive and quite specific, since it is not
en by other phosphate compounds (8).

Chemical analysis as well as histochemical staining reactions for meta-
omatic corpuscles shows that metaphosphate is a normal constituent of
yeast cell. It disappears when the yeast is grown in a medium poor in
osphate (phosphorus-starved yeast). Its synthesis requires phosphate,
assium ions (9), and a substrate which can be metabolized. Meta-
osphate can be formed anaerobically as well as aerobically. Poisons which
p fermentation (iodoacetate, fluoride) also stop the synthesis of meta-
osphate. Sodium azide under anaerobic conditions, with glucose as sub-
ate, at a concentration which does not decrease the rate of fermentation
5×10^{-4} M) stops the synthesis of metaphosphate. The largest amount
metaphosphate is synthesized by yeast previously starved with respect
phosphorus (2, 3). In such yeast the metaphosphate accounts for most
the phosphorus taken up by the cells from the medium.

t is of interest that the bonds in metaphosphate are of the energy-rich
e and that it is present in yeast as a polymer. Its property of combining
h protein *in vitro*, even to form a crystalline product (10), is also of great
erest.

The experiments reported in this paper show that two types of meta-
osphate occur in yeast, one which is soluble in cold trichloroacetic acid
ferred to as soluble metaphosphate), the other which can be extracted
y by hot trichloroacetic acid (referred to as insoluble metaphosphate)

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or by alkali. These two fractions are also physiologically distinct, the latt
being metabolically more active in the cell than the former. The insolub
metaphosphate is rapidly and reversibly transformed to orthophosphate
follows:

$$\text{Orthophosphate} \underset{\text{(II)}}{\overset{\text{(I)}}{\rightleftarrows}} \text{insoluble metaphosphate}$$

These are over-all reactions. The enzymes and number of steps involve
are not yet known. Reaction (I) occurs when yeast is metabolizing glucos
while reaction (II) can be readily observed when yeast metabolism is stopp
by low temperature. The physiological difference between the soluble a
the insoluble metaphosphate can also be demonstrated by studying the ra
of phosphorus turnover in the different phosphate fractions of the yea
(11).

EXPERIMENTAL

Preparation of Yeast—Phosphorus-starved yeast was prepared by gro
ing it in the following medium: sucrose 100 gm., succinic acid 20 gm., K_2S
5 gm., $(NH_4)_2SO_4$ 2 gm., $MgSO_4 \cdot 7H_2O$ 0.25 gm., $Fe_2(SO_4)_3$ 0.15 gm
$ZnSO_4 \cdot 7H_2O$ 0.02 gm., $MnSO_4 \cdot 4H_2O$ 4 mg., $CuSO_4 \cdot 5H_2O$ 100 mg., inos
tol 100 mg., thiamine hydrochloride 2 mg., Ca pantothenate 2 mg., nic
tinic acid 2 mg., pyridoxine 2 mg., biotin 20 γ, and water to 10 liter
The succinic acid is dissolved separately and is neutralized with 10 ml.
N NaOH and concentrated ammonia to pH 5.

The medium is seeded with 30 gm. of bakers' yeast, incubated at 29
and grown overnight with aeration. Yeast harvested from such a cultu
and washed with water is completely free of metachromatic material an
contains no metaphosphate. The yeast is separated in a Sharples centr
fuge, and 20 gm. are suspended in 1 liter of a medium containing 2 per cer
glucose in M/30 KH_2PO_4 and strongly aerated at 29°. Metachromat
material can be detected cytochemically after 5 minutes, and it increas
over a period of 1.5 to 2 hours.

Qualitative and Quantitative Determination of Metaphosphate—It is we
known that metaphosphate has the property of precipitating proteins, an
that it is completely hydrolyzed after 7 minutes in N HCl at 100°. Tw
other properties are very useful, the insolubility of barium metaphosphate
low pH values and the metachromatic reaction. The precipitate of bariu
metaphosphate is gelatinous; when the concentration of metaphosphate
low, flocculation can be effected by scratching the wall of the containe

Barium precipitation was used for the separation of metaphosphat
While with pure hexametaphosphate precipitation is complete at pH 2.
with yeast extracts (10 per cent trichloroacetic acid in the cold) it is so onl

hen the pH is increased to 4.5. The determination was made as follows: ml. of the trichloroacetic acid (TCA) solution containing from 30 to 300 of metaphosphoric P were neutralized with NaOH to about pH 4.5; 1 l. of 5 M acetate buffer, pH 4.5, was added, followed by 1 ml. of a saturated lution of $Ba(NO_3)_2$. This mixture was shaken overnight in a cold room. he precipitate was centrifuged down, washed with 5 ml. of water, dislved in 2 ml. of N HCl, and diluted with water to a convenient volume. this solution the inorganic P formed after 7 minutes of hydrolysis in N Cl at 100° was found to be equal to the total P after ashing. Tests for entose and ketose were negative. Inorganic pyrophosphate was not dectable with zinc acetate at pH 3.7 as the test reagent. Therefore, the total 7 minute P" precipitated with Ba at pH 4.5 may be assumed to be metaosphate.

In one experiment the metaphosphate extracted from yeast with cold TCA as precipitated with barium at pH 2.5. The low pH was used in order to oid adsorption of foreign materials on the gelatinous precipitate. The ied precipitate contained 19.6 per cent P. A precipitate obtained under e same conditions with pure hexametaphosphate gave 19.8 per cent P. he theoretical value is 21 per cent.

The metachromatic test has been used for the cytochemical detection of etaphosphate (2, 3). It can also be used in solution as an ordinary chemal test (8), provided interfering substances are removed. For this purpose e TCA extract at a pH of about 4 is treated with 0.25 volume of 5 per cent $b(NO_3)_2$ in order to precipitate metaphosphate. The precipitate is washed d suspended in about 10 to 20 volumes of water and acidified with HCl about pH 2. H_2S is passed through the suspension for half an hour and e PbS is centrifuged off. The supernatant fluid which must be clear olloidal PbS adsorbs metaphosphate) is aerated. The solution is added op by drop to 2 ml. of a solution of toluidine blue (30 mg. per liter). A ange of color from blue to purple indicates the presence of metaphosphate. ith this test metaphosphate can be detected in a concentration of 10^{-4} M.

Soluble Fraction—It has been shown by Macfarlane (6) that the amount of thophosphate present in the acid-soluble fraction of yeast depends upon e physiological state of the cell. It decreases when fermentation starts d increases rapidly to the initial value when fermentation stops. For is reason all analyses were performed on yeast in a non-fermenting state. order to produce this condition the cells, after washing, are kept in susension in water at room temperature for about 10 minutes. After this eatment the extraction is carried out as follows: About 1 gm. of yeast (aproximately 250 mg. of dry weight) is centrifuged in a 50 ml. tube. The east is extracted three times for 1 hour each with 4 ml. of 10 per cent TCA hile being shaken in a cold room (0–4°). The three extracts are pooled, diluted to 25 ml. with water, and used for the determination of the differe fractions: orthophosphate, phosphate formed in 7 minutes in N HCl at 10 (7 minute P), metaphosphate, and total P. It was found that the fir second, and third extracts contained respectively about 90, 9, and less tha 1 per cent of the total acid-extractable phosphorus.

When the extraction is made with cold 5 per cent instead of 10 per cen TCA, significantly less P is extracted, and further extraction with 10 p cent TCA gives a phosphorus fraction which is chiefly metaphosphate. Th incompleteness of the extraction with 5 per cent TCA had been observed b Macfarlane (6). The orthophosphate is completely extracted with 5 p cent TCA. Examples are given in Table I. Three extractions with 1 per cent TCA, as outlined above, were used in all further experiments.

TABLE I

Extraction of Metaphosphate from Yeast

Yeast extracted twice with 5 per cent trichloroacetic acid followed by two ex tractions of residue with 10 per cent trichloroacetic acid. The results are expresse in gm. of P per 100 gm. of dry yeast.

Type of yeast	Extracted twice with TCA	Ortho-phosphate	7 min. P	Metaphosphate, B ppt. at pH 4.5
	per cent			
Phosphorus-starved yeast after 90 min. with glucose and KH_2PO_4	5	0.423	1.03	Not tested
	10	0.015	0.38	" "
Unstarved yeast after 10 min. with glucose, no phosphate	5	0.156	0.11	0.00
	10	0.00	0.00	0.00
Unstarved yeast after 120 min. with glucose and KH_2PO_4	5	0.26	0.17	0.06
	10	0.00	0.16	0.13

Insoluble Fraction—The residue of the cold TCA extract is treated for th removal of phospholipides according to the method of Schneider (12). I is extracted once with 10 ml. of ethanol at room temperature and twice wit 10 ml. of ethanol-ether mixture (3:1) in a bath at 90° for 3 minutes. Nuclei acid and other phosphate compounds are extracted from the residue a follows: It is first treated with 10 ml. of 5 per cent TCA at room temperature and then twice with the same volume of TCA at 95°. The combine extracts are then treated with steam for 4 minutes to remove the TCA and finally diluted to 50 ml. Part of this solution, diluted ten to twent times, is used for the determination of the absorption of ultraviolet ligh (260 mμ) in the Beckman photoelectric spectrophotometer. The rest o the solution is used for phosphate determination.

Examples are given in Table II. A commercial sample of yeast ribo nucleic acid (Sample 1) and ribonucleic acid reprecipitated with HCl (Sampl

), both treated at 95° with 5 per cent TCA, are included for comparison. t will be seen in Samples 3, 4, and 5 that phosphorus starvation decreases oth the amount of P and purines and pyrimidines in such a manner that ne ratio of the extinction at 260 mμ to phosphorus remains practically onstant. As this ratio is the same as that found in nucleic acid, it indicates nat nucleic acid is the sole important phosphorus-containing material in nis fraction. This ratio is changed very markedly when phosphorus-tarved yeast is incubated for 1 hour in the presence of glucose and KH_2PO_4 Samples 6 and 7), owing to the presence of an excess of phosphorus in the nucleic acid" fraction.

TABLE II

omparison of Ratio (Purines + Pyrimidines)/(Total P) in Yeast Nucleic Acid and in Hot Trichloroacetic Acid Extracts from Different Yeasts

$\epsilon = \log I_0/I$ for a width of 1 cm.

mple No.	Material tested	Total P per ml.	$\epsilon_{260\ m\mu}$	$\frac{\epsilon_{260\ m\mu}}{P}$
		mM × *10*$^{-4}$		× *10*^{3}
1	Ribonucleic acid	1.23	1.31	10.6
2	Reprecipitated ribonucleic acid	0.57	0.61	10.7
3	Extract of bakers' yeast	1.58	1.70	10.7
4	Same as (3) after phosphorus-starvation (1st starvation)	0.96	0.87	9.5
5	Same as (4) (2nd starvation)	0.61	0.59	9.7
6	Yeast of (5) after incubation in glucose + KH_2PO_4	3.5	0.73	2.1
7	Phosphorus-starved yeast after incubation in glucose + KH_2PO_4	1.65	0.78	4.7

This phosphorus is partly orthophosphate, partly easily hydrolyzable hosphate. For example, of 440 mg. of "nucleic acid" P per 100 gm. of east, 150 mg. were orthophosphate and 70 mg. were 7 minute P.[1] Since rthophosphate had already been extracted with cold 10 per cent TCA, it eems probable that partial hydrolysis of some labile phosphorus compound ccurred during extraction with hot 5 per cent TCA. By deducting the rthophosphate plus 7 minute P from the total P, the ratio, $\epsilon_{260\ m\mu}$ to P, was ound to be in agreement with that for nucleic acid. Examples for this will e given later.

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[1] A correction was applied in all cases for a small quantity of orthophosphate hich is formed as a result of the hydrolysis of nucleic acid with N HCl at 100° uring the 7 minute P determination. This correction is based on the known rate hydrolysis of ribonucleic acid under the same conditions (2) and is applied as llows: 1.8 × (14 minute P minus 7 minute P).

Nature of Insoluble 7 Minute P—In order to study the nature of th material it was necessary to find a method of extraction which would prever hydrolysis. The best results were obtained by suspending the residue of th lipide extraction in water and adjusting the pH to between 8 and 9 wit NaOH. After shaking for 1 hour at room temperature, the residue wa removed by centrifugation and reextracted for a second and third time Another aliquot was extracted with hot 5 per cent TCA as described in th preceding section. The results (Table III) show that the alkaline extract contained 7 minute P which was almost completely precipitated by bariu nitrate at pH 4.5. Orthophosphate was not detectable in appreciable quantity. The total 7 minute P extracted with alkali was in good agreement wit the value obtained with the hot TCA extraction. The alkaline extrac also showed a positive metachromatic reaction. It therefore seems clea that the insoluble 7 minute P consists largely, if not entirely, of metaphosphate.

TABLE III

Comparison of Extraction of Insoluble Metaphosphate with Alkali and with H Trichloroacetic Acid

The results are expressed in gm. of P per 100 gm. of dry yeast.

Method of extraction	Extraction	7 min. P*	Metaphosphate, Ba ppt. at pH 4.5
NaOH, pH 8	1st	0.236	0.212
	2nd	0.018	0.010
	3rd	0.002	
Total		0.256	0.222
NaOH, pH 9	1st	0.252	0.213
	2nd	0.016	0.006
	3rd	0.001	
Total		0.269	0.219
Hot TCA		0.28†	

* Corrected for hydrolysis of nucleic acid. See foot-note 1.

† Orthophosphate plus 7 minute P.

It seemed possible that the insoluble metaphosphate was an artifact du to the formation of a protein-metaphosphate complex during the extractio of yeast with cold TCA. This, however, does not appear to be the case since TCA, in contrast to other acids, when mixed with protein and meta phosphate gives a precipitate which contains very little phosphorus. Whe 1 ml. of rabbit serum was mixed with 5 ml. of 1 per cent sodium hexameta hosphate and 2 ml. of 20 per cent acetic acid, an abundant precipitate was ormed. After washing this precipitate twice with 10 per cent acetic acid was found to contain 3.4 mg. of phosphorus. When the same experiient was performed with 10 per cent TCA instead of acetic acid, the preipitate contained only 0.1 mg. of P. Probably, because of the denaturation f protein, no protein-metaphosphate complex is formed in the presence of 'CA.

The whole procedure for the quantitative determination of the two fracons of metaphosphate can be summarized as follows: 1 gm. of yeast is xtracted three times with cold 10 per cent TCA. From this extract the oluble metaphosphate is precipitated at pH 4.5 with barium nitrate. The esidue of this extraction is treated with a mixture of ethanol-ether to remove ie phospholipides and extracted twice with 5 per cent TCA at 95°. The rthophosphate plus the 7 minute labile phosphate of this fraction, after orrection for the slight hydrolysis of nucleic acid, is taken as the amount of isoluble metaphosphate.

Physiological Behavior of Soluble and Insoluble Metaphosphate—In order to tudy the behavior of the two metaphosphate fractions, their fate in the ving cell was followed under different conditions.

Experiment 1—40 gm. of phosphorus-starved yeast were suspended in 1.8 ters of a medium containing 40 gm. of glucose and maintained at 27–28° ith constant aeration. After the initial sample (Sample 1) was taken, 180 il. of M/3 KH_2PO_4 were added. The yeast was incubated in this medium for hour (Sample 2). After this the yeast was kept in the cold to stop further ynthesis of metaphosphate, washed, and resuspended in the phosphateree growth medium previously described. A last sample was taken after 0 minutes of incubation at 27° under aeration (Sample 3). The results Table IV) are given in per cent of P per initial dry weight of the yeast. 'here was no appreciable growth in this experiment. At the beginning the east contained a very small amount of acid-soluble P and no detectable oluble metaphosphate. The fraction insoluble in cold TCA consisted alrost entirely of nucleic acid. During incubation with glucose and phoshate large amounts of soluble as well as insoluble metaphosphate were ormed. In the third period the most striking fact was the marked decrease i the quantity of insoluble metaphosphate and a parallel increase in orthohosphate without any change in the soluble metaphosphate.

Experiment 2—The experiment was similar to the preceding one except hat after the metaphosphate synthesis the yeast was kept in the cold for bout 3 hours in the glucose-phosphate medium (Sample 3). It was then icubated in the growth medium containing glucose but no phosphate. The esults are given in Table V. It is evident that keeping the yeast in the cold eads to a decrease in the amount of insoluble metaphosphate and an in-

Table IV

Formation and Disappearance of Insoluble Metaphosphate

For description of samples see the text. The results are expressed in gm. of per 100 gm. of dry yeast.

Sample No.	Time	Conditions	Soluble in cold trichloroacetic acid				Insoluble in co trichloroaceti acid	
			Total P	Metaphosphate	7 min. P	Orthophosphate	Total P	Met phos phat
	min.							
1	0	Phosphorus-starved yeast	0.09	0.00	0.00	0.02	0.232	0.0
2	60	After incubation with glucose and KH_2PO_4 at 27°	1.99	1.05	1.34	0.433	0.750	0.4
3	120	Kept at 0°, washed and resuspended for 10 min. in growth medium without phosphate at 27°	2.21	1.22	1.42	0.684	0.54	0.2

Table V

Changes in Metaphosphate Fractions of Yeast

The results are expressed in gm. of P per 100 gm. of dry yeast.

Sample No.	Time	Conditions	Soluble in cold trichloroacetic acid				Insoluble in cold trichl oacetic acid		
			Total P	Metaphosphate	7 min. P	Orthophosphate	Total P	Metaphosphate	ϵ_{260} / Nucl acid
	min.								× 1
1	0	Phosphorus-starved yeast	0.077	0.00	0.01	0.027	0.198	0.012	9.
2	75	After incubation with glucose and KH_2PO_4 at 28°	1.80	0.94	1.36	0.347	0.669	0.459	9.
3	240	Kept at 0° in same medium	1.96	0.94	1.31	0.456	0.454	0.246	8.
4	265	Incubated in growth medium without phosphate for 25 min. at 28°	1.85	0.93	1.27	0.405	0.518	0.328	9.

* Nucleic acid P = (total P) − (metaphosphate) in mM per ml. (*cf.* Table II)

crease in orthophosphate. As the soluble metaphosphate did not chang it is apparent that the orthophosphate was derived from the insoluble met phosphate. In the phosphate-free growth medium (Sample 4) there occurr a resynthesis of insoluble metaphosphate.

Experiment 3—This experiment differed from the preceding one in th n the last period the yeast was suspended in a medium containing glucose ut no nitrogen source. The results are given in Table VI. Here again, ne finds a decrease in insoluble metaphosphate and an increase in ortho-

TABLE VI

Resynthesis of Insoluble Metaphosphate

The results are expressed in gm. of P per 100 gm. of dry yeast.

ample No.	Time	Conditions	Soluble in cold trichloroacetic acid			Insoluble in cold trichloroacetic acid
			Metaphosphate	7 min. P	Orthophosphate	Metaphosphate
	min.					
1	0	Metaphosphate-rich yeast	0.93	1.31	0.49	0.390
2	120	Kept at 0° with glucose and KH_2PO_4	0.90	1.36	0.60	0.310
3	190	Washed and resuspended for 70 min. at 28° in medium containing glucose but no phosphate or nitrogen source	0.79	1.1	0.20	0.400

TABLE VII

Relation of Metaphosphate to Synthesis of Nucleic Acid

The results are expressed in gm. of P per 100 gm. of dry yeast (initial weight be-ore growth).

ample No.	Time	Conditions	Soluble in cold trichloroacetic acid				Insoluble in cold trichloroacetic acid		
			Total P	Ba ppt. of metaphosphate		Orthophosphate	Total P	Metaphosphate	Nucleic acid P
				Total P	7 min. P				
	min.								
1	0	Phosphorus-starved yeast	0.066	0	0	0.015	0.157	0.016	0.141
2	60	After incubation at 28° with glucose + KH_2PO_4	2.24	1.22	1.22	0.475	0.648	0.484	0.164
3	240	After incubation in growth medium without phosphate	2.10	1.22	1.21	0.340	0.645	0.178	0.467
4	420	Prolonged growth	1.21	0.44	0.43	0.210	1.30	0.288	1.012

hosphate during the period when the suspension of yeast in glucose and KH_2PO_4 was kept in the cold. Sample 3 shows that the formation of in-oluble metaphosphate at the expense of internal P does not need an external

source of nitrogen, exactly as in the case of the synthesis of this compo
in the presence of glucose and KH_2PO_4. However, in the absence of nitro
the yeast lost a considerable amount of P to the medium. The amoun
soluble metaphosphate and orthophosphate decreased, the latter more t
one would expect from the increase in insoluble metaphosphate.

Experiment 4—In this experiment the yeast which had previously
cumulated metaphosphate was allowed to grow in the phosphate-free
otherwise complete medium. The results are presented in Table VII.
amount of phosphorus in the different fractions is expressed on the basi
the initial dry weight of the yeast. When growth began (Sample 3),
quantity of nucleic acid P (total insoluble P minus insoluble metaphospha
increased. Since no phosphate was present in the medium, the phospho
came from that contained in the cells. It can be seen that at first this

TABLE VIII

Synthesis of Soluble and Insoluble Metaphosphate in Normal and Phosphorus-Star
Yeast

The results are expressed in gm. of P per 100 gm. of dry yeast.

Conditions	Soluble in cold trichloroacetic acid			Insoluble in cold trichloroacetic acid	Tota metaph phat
	Metaphosphate	7 min. P	Orthophosphate	Metaphosphate	
Phosphorus-starved yeast	0	0.01	0.027	0.012	0.01
Normal yeast	0	0.109	0.156	0.039	0.03
After incubation with glucose + KH_2PO_4 for 120 min.					
Phosphorus-starved yeast	0.940	1.36	0.347	0.459	1.39
Normal yeast	0.190	0.334	0.264	0.585	0.77

crease was accompanied by a corresponding decrease in the insoluble me
phosphate of the yeast, while the soluble metaphosphate remained consta
As growth continued the absolute amount of nucleic acid continued to
crease, so that at the end of the experiment (Sample 4) when the numb
of cells had approximately doubled, the nucleic acid P had increased m
than 7-fold. After the initial decrease, the insoluble metaphosphate
creased slightly, while the soluble metaphosphate was rapidly used
At the beginning of growth the demand for phosphorus was satisfied
the insoluble metaphosphate and orthophosphate of the cells. Later, wh
a low level of the insoluble metaphosphate had been reached, the solu
metaphosphate was used as a source of phosphorus. This experime
however, does not necessarily indicate that metaphosphate is a normal int
mediate product in the synthesis of nucleic acid.

Experiment 5—It has already been reported (2) that the accumulation aphosphate is greatest when synthesis occurs after a period of phosphorus rvation. It was of interest to compare the amounts of soluble and in- uble metaphosphate synthesized by normal and phosphorus-starved yeast s. The results of such an experiment are presented in Table VIII. en the two series of values are compared, it may be seen that the total taphosphate accumulated by starved yeast (1.39 per cent P) is greater n that formed in normal yeast (0.77 per cent P). This difference is due a large accumulation of soluble metaphosphate in phosphorus-starved st; insoluble metaphosphate is synthesized by both normal and starved s to approximately the same extent.

SUMMARY

. Two distinct fractions of metaphosphate occur in yeast. One, termed luble" metaphosphate, can be extracted with cold 10 per cent trichloro- tic acid. The other, referred to as "insoluble" metaphosphate, can be racted (together with nucleic acid) by alkali or by hot 5 per cent trichloro- tic acid. Methods for the quantitative determination of the two frac- ns are described.

2. Solutions of both types of metaphosphate give a metachromatic reac- n with toluidine blue.

3. The insoluble metaphosphate is metabolically more active than the uble one and is rapidly and reversibly transformed to orthophosphate in cell. When yeast which has previously accumulated metaphosphate is owed to grow in a medium without phosphate, nucleic acid synthesis is ompanied at first by a disappearance of insoluble metaphosphate and then tinues at the expense of the soluble metaphosphate.

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N THE POSSIBLE UTILIZATION OF SULFATE SULFUR BY THE SUCKLING RAT FOR THE SYNTHESIS OF CHONDROITIN SULFATE AS INDICATED BY THE USE OF RADIOACTIVE SULFUR

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It has been shown that the animal organism cannot avail itself of admin- stered sulfate sulfur for the synthesis of cystine *in vivo* (1). The data of Singher and Marinelli (2) and our own data (3), though showing an accumula- ion of S^{35}, given as sulfate, in bone and bone marrow, still do not indicate hat the sulfur has been incorporated in any new compound. That sulfate ulfur can be utilized in the synthesis of urinary ethereal sulfates *in vivo* as been demonstrated (4–7). It may be that sulfate of exogenous origin an also combine in similar fashion through ester linkages in an animal organism to form compounds like chondroitin sulfate, mucoitin sulfate, heparin, nd the like. These might be retained in an animal's tissues for some time. o test this possiblity the concentration of S^{35}, given as sodium sulfate by ntraperitoneal injection, was followed in the knee joint cartilage of suckling ats. An attempt was also made to determine whether or not the S^{35} present n the cartilage was present therein as chondroitin sulfate.

EXPERIMENTAL

In the first series of experiments 7 day-old suckling rats of both sexes from he colony of Professor E. V. McCollum were injected intraperitoneally vith 0.2 mg. of sodium sulfate, containing S^{35} (2.26×10^5 counts per min- te) in 0.1 ml. of water.[1] All the animals were carefully observed for about 0 minutes after injection and discarded if there was evidence of leakage. fter injection the animals were returned to the mother.

The animals were sacrificed by decapitation. Blood was collected by hold- ng the decapitated animal over a tared, small porcelain evaporating dish.

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[1] The S^{35} used in this investigation was supplied by the Clinton Laboratories, onsanto Chemical Company, and obtained on allocation from the United States tomic Energy Commission.

The evaporating dish with the blood was covered with a tared watch-gla and weighed immediately. The blood samples were oxidized with the r agent of Denis (8). Before precipitation of the sulfate as barium sulfa 5 ml. of a 0.05 N solution of sodium sulfate were added as carrier.

The knee joints were freed of muscle, tendons, and sheath. After rinsi in distilled water and blotting with absorbent paper, they were weighed i mediately. They were then ignited to red heat with about 20 mg. of a hydrous sodium carbonate in covered quartz evaporating dishes. On coo ing, the residue was taken up in a slight excess of 20 per cent hydrochlor acid and filtered through acid-resistant filter paper. The quartz dish w rinsed with 5 ml. of 0.05 N sodium sulfate solution and repeatedly wit small amounts of distilled water. All rinsings were passed through th filter paper in turn. The sulfate in the filtrate was precipitated as bariu sulfate.

All barium sulfate samples were isolated by centrifugation and, after wash ing with distilled water in the centrifuge tubes, transferred as slurries in 7 per cent ethanol to tared counting cups (9).

The activity of each sample was determined by the use of a 2.8 mg. pe sq. cm. mica end window Geiger-Müller tube (Victoreen), and a Cyclotro Specialties scaler. At least two separate determinations of the radio activity of each sample were made for a period of time sufficiently long t obtain a precision of about 2 per cent. All values were corrected for deca and self-absorption.

In the second series of experiments suckling rats were injected intra peritoneally with 0.1 mg. of labeled sodium sulfate (S^{35}, 1.13×10^5 counts pe minute) in 0.05 ml. of water. Four rats were thus injected when 3 days ol four when 5 days old, four when 7 days old, and four when 9 days old. A these rats were sacrificed 24 hours after injection. Blood and cartilage we analyzed as above.

Some of the animals that were inadequately injected were set aside an sacrificed either 24 hours or 48 hours later. One cleaned knee joint fro each animal was placed in each of two pools. One of these pools of cartilag was then fused with sodium carbonate as described above. The cartilage the second pool was treated according to the method of Bray *et al.* (10) f the isolation of chondroitin sulfate, except that instead of isolating the cho droitin sulfate the filtered clear solution of the barium salt of chondroit sulfate was exhaustively hydrolyzed with concentrated hydrochloric aci On cooling and dilution of the hydrolysate, barium sulfate was precipitate To insure more complete precipitation of the sulfate from chondroitin su fate, 5 ml. of 0.05 N sodium sulfate and 5 ml. of 5 per cent barium chlori solution were added to the cool dilute hydrolysate. The barium sulfa was isolated by centrifugation and its activity determined as indicat above.

To determine the fraction of the dose retained by suckling rats after adinistration of 0.1 mg. of labeled sodium sulfate (S^{35}, 1.13 × 10^5 counts per inute) eight rats, 7 days old, were injected intraperitoneally. Four of ese rats were sacrificed by decapitation 24 hours later; the remaining four ts, 48 hours later. Decapitation was done over a beaker containing 50 l. of 10 per cent sodium hydroxide. The entire carcass of each rat was ydrolyzed for approximately 8 hours in this sodium hydroxide solution on steam bath. The hydrolysate (residual bone was easily pulverized with glass stirring rod to a fine powder) was diluted, while still hot, to 200 ml.

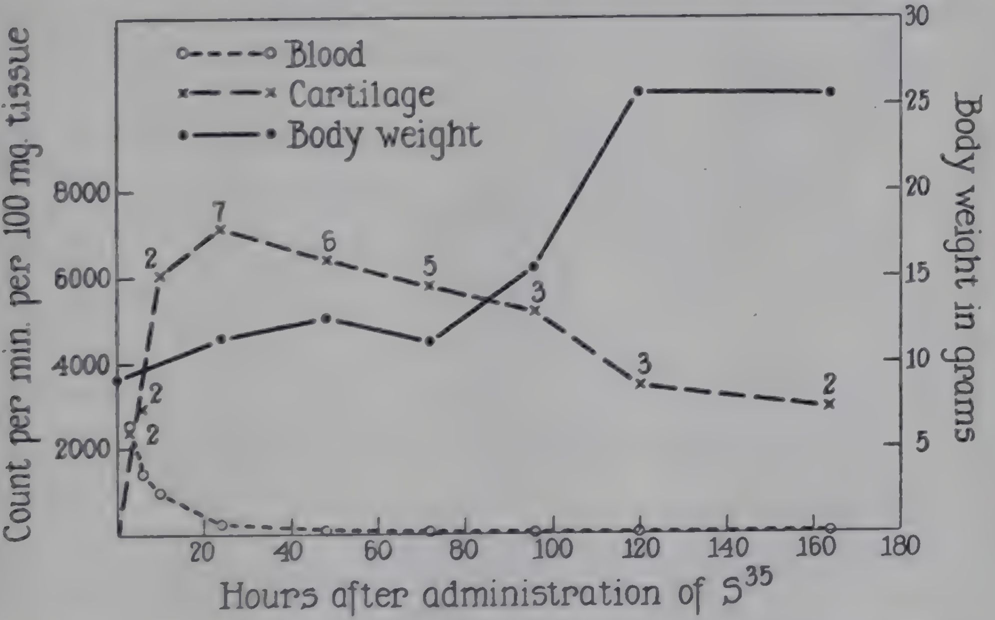

FIG. 1. Retention of S^{35} by the blood and articular cartilage of suckling rats. he rats were 7 days old at the time each was given 0.2 mg. of labeled sodium sulate (2.26 × 10^5 counts per minute) by intraperitoneal injection. The number above ny point along the curve for S^{35} in cartilage indicates the number of animals used arrive at the value of the point and the corresponding points on the other curves.

fter mixing well to suspend the bone powder, 5 ml. aliquots were transerred to nickel crucibles and dried with 4 gm. of anhydrous sodium carbonte in a drying oven at 110–120°. The dried material was mixed with soium peroxide and oxidized according to the directions of Bailey (11).

RESULTS AND DISCUSSION

Observations on the change in the concentration of S^{35} with time in the artilage of the knee joints after intraperitoneal administration of labeled odium sulfate to 7 day-old suckling rats are summarized in Fig. 1. Examiation of Fig. 1 shows that there is a progressive increase in the concentraon of S^{35} until the 24th hour after the injection of the labeled sodium sulfate. uring the same period of time the blood concentration of S^{35} falls rapidly. fter the 24th hour the S^{35} appears to be slowly lost from the cartilage.

The decrease in the concentration of the S^{35} in the cartilage is actually ev
less rapid than that indicated in Fig. 1. From Fig. 2, in which the activit
of S^{35} per cartilage sample is plotted against time, it is evident that th
apparent loss is probably mostly a dilution effect due to the increase in th
weight of the cartilage of the growing animals. It is realized that the weigh
of cartilage samples were not the weights of cartilage actually present in th
knee joints: some of the cartilage was trimmed away in cleaning the kne
joints. But, even with this consideration in mind, it is suggested that th
curve of the activity of S^{35} found in the cartilage samples with increasing tim
Fig. 2, indicates the possibility that the S^{35} was retained in some form in th
cartilage without appreciable loss between the 24th and 164th hours aft

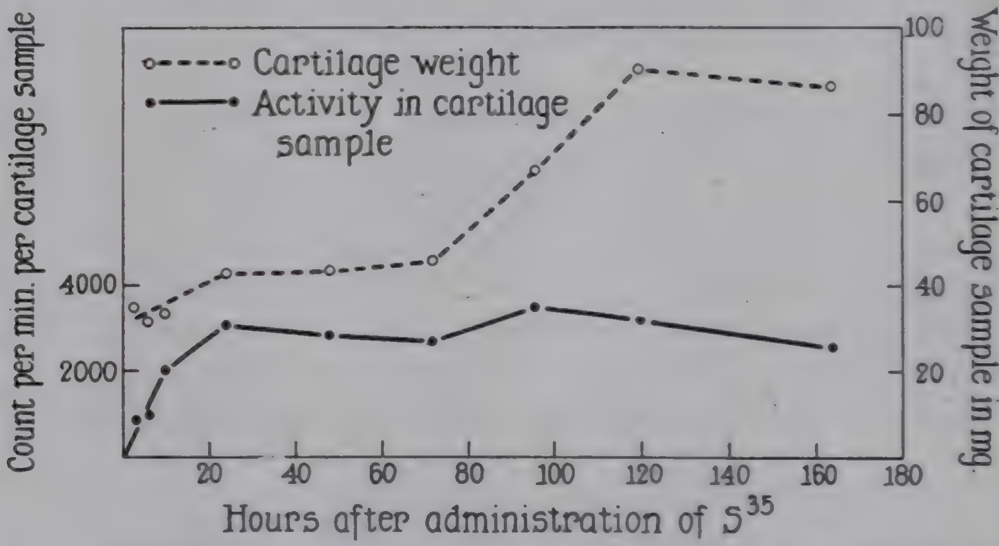

FIG. 2. Concentration of S^{35} per sample of articular cartilage obtained from th
knee joints of suckling rats. The rats were 7 days old at the time each was given 0.
mg. of labeled sodium sulfate (2.26×10^5 counts per minute) by intraperitoneal in
jection. The value of each point is the average value obtained on the number o
animals shown in Fig. 1.

administration of the labeled sodium sulfate. It is suggested that the ap
parent decrease, Fig. 1, in the S^{35} concentration, 24th to 164th hour, is pr
marily a result of dilution with newly formed cartilage.

The experiment summarized in Table I was an attempt to determin
whether the rate of uptake of S^{35}, given as sodium sulfate, changed markedl
with the age of the suckling rat. From Table I, Column 6, it would appea
that the rate of uptake decreases with age in rats 3 to 9 days old. A ca
culation made on the basis of difference in weight of the animals at th
different ages, Column 7, Table I, however, suggests that there is little diffe
ence in the rate of uptake of the S^{35} by the cartilage. Similar calculation
made for the blood did not give values approaching a constant.

The experiments cited thus far only suggested the possibility that sulfa fur might be utilized by the suckling rat in the synthesis of chondroitin fate. As a test of this possibility, the cartilage of the knee joints of in- equately injected animals was divided into two pools on two separate

TABLE I

in Blood and Cartilage of Suckling Rats Injected Intraperitoneally at Different Ages with Labeled Sodium Sulfate

Each rat was given 0.1 mg. of sodium sulfate, containing S^{35} (1.13×10^5 counts r minute) in 0.05 ml. of water. The rats were sacrificed 24 hours later.

Rat No. (1)	Age (2)	Body weight (3)	Blood (4)	$W \times A$* (5)	Cartilage (6)	$W \times A$* (7)
	days	*gm.*	*counts per min. per 100 mg.*		*counts per min. 100 per mg.*	
1	3	9.7	172		8470	
2		9.0	167		7375	
3		8.2	318		3395	
4		9.0	285		5850	
Average..	....	9.0	235	2115	6272	56,448
5	5	11.4	63		4425	
6		8.3	61		4310	
7		11.3	50		4840	
8		10.0	41		5260	
Average..	....	10.3	54	556	4709	48,502
9	7	13.0	30		3350	
10		14.0	29		3285	
11		15.4	32		3250	
12		11.8	55		4660	
Average..	....	13.6	37	503	3636	49,449
13	9	16.5	68		3460	
14		13.5	63		3485	
15		15.2	88		3880	
16		15.4	59		3590	
Average........		15.2	69	1049	3604	54,780

* Body weight × activity in 100 mg. of cartilage or 100 mg. of blood.

ccasions, and analyzed as described in the experimental section. The re- ılts of this experiment, shown in Table II, suggest that the suckling rat ay use exogenous sulfate sulfur in the synthesis of chondroitin sulfate and ıat the major portion, if not all, of the sulfate sulfur retained in the cartilage ay thus be used. The data in Table II are not as yet unequivocal proof

TABLE II

Concentration of S^{35} in Chondroitin Sulfate after Intraperitoneal Administratio Labeled Sodium Sulfate

Experiment No.	Activity per 100 mg. cartilage after sodium carbonate fusion	Activity in partially purified c droitin sulfate from 100 mg. car
	counts per min.	*counts per min.*
1	9380 (227)	9200 (235)
2	3660 (178)	3615 (177)

The rats in Experiment 1 were killed 24 hours after an attempt to inject 2.2 10^5 counts per minute per animal; those in Experiment 2, 48 hours after an atter to inject 1.13 × 10^5 counts per minute per animal. Some of the animals used these experiments received an additional injection immediately after the first tempt was found to be unsuccessful. The figures in parentheses are the act weights in mg. of the cartilage in the samples.

A test was made of the possibility that inorganic sulfur was carried along in partial purification of the chondroitin sulfate. To each of two pools of cartila from uninjected suckling rats, 1 mg. of sodium sulfate containing S^{35} (945 cou per minute) was added. The sulfur of the partially purified chondroitin sulfate w isolated as $BaSO_4$ and the activity of the latter determined. In one instance counts per minute and, in the second, 32 counts per minute were observed.

TABLE III

Concentration of S^{35} in Carcass of Suckling Rat 24 and 48 Hours after Intraperiton Administration of Labeled Sodium Sulfate

Each rat, when 7 days old, received 0.1 mg. of sodium sulfate, containing (1.13 × 10^5 counts per minute).

Rat No. (1)	Time after injection (2)	Body weight (3)	S^{35} in whole carcass (4)	S^{35} per 100 mg. carcass (5)	S^{35} excreted (6)	
	hrs.	*gm.*	*counts per min.*	*counts per min.*	*counts per min.*	*per cen*
1	24	13.6	35,065	258	77,935	68.9
2		14.0	38,190	273	74,810	66.2
3		15.4	35,390	230	77,610	68.6
4		12.8	36,340	284	76,660	67.8
Average		14.00	36,246	261	76,754	67.8
5	48	17.4	21,777	125	91,223	80.7
6		17.7	22,176	125	90,824	80.3
7		17.6	25,162	143	87,838	77.7
8		18.2	28,493	157	84,507	74.8
Average		17.72	24,402	138	88,598	78.3

that it is chondroitin sulfate which holds the S^{35}. They are strongly sugg tive of this possibility, but it is still necessary to isolate and characte chondroitin sulfate to establish the fact completely.

Vith the demonstration of the retention of sulfate sulfur by the cartilage, question arose as to the amount of S^{35} which these animals excreted after ninistration of labeled sodium sulfate. An answer was sought indirectly, e rats of the suckling age employed could not be isolated very well for collection of urine and feces. The activity of S^{35} in the carcass at the end the 24th and 48th hours was determined instead. The difference be- en the amount detected in the carcass and the amount of S^{35} adminis- ed was assumed to be the amount excreted. The results of this ex- iment are summarized in Table III. The suckling rat at 7 days of age pears to excrete about as much of a 0.1 mg. dose of sodium sulfate as an ılt rat excretes of a 1 mg. dose (3). In the suckling rats used only 20 to per cent of the S^{35} administered as sodium sulfate could be detected in the rcass after a lapse of 48 hours.

It is of interest to compare the data in Table III, Column 5, for the rats crificed after 24 hours with the data in Table I, Column 6, for the 7 day- l rats. From these figures it appears that in 7 day-old rats, 24 hours after ection, the average concentration of S^{35} in the carcass is 261 counts per nute per 100 mg., whereas that in the cartilage is 3636 counts per minute r 100 mg. The average concentration of S^{35} in the cartilage is thus found be about 14 times as great as the average concentration in the whole rcass.

SUMMARY

Suckling rats, 7 days of age, were found after 48 hours to have retained to 25 per cent of the S^{35} contained in a 0.1 mg. dose of labeled sodium lfate.

Of the S^{35} retained by the suckling rat, 7 days of age, an amount many nes that present in the blood and about 14 times that present in the hole carcass at the 24th hour was found in the cartilage of the knee joints. n increase in the concentration of the S^{35} in the cartilage was observed up to e 24th hour after administration of the labeled sodium sulfate. The sub- quent decrease in the S^{35} concentration up to the 164th hour was slight.

Data are presented which suggest that the rate of uptake of S^{35}, admin- ered as sodium sulfate, may not be markedly different in suckling rats o 9 days of age.

On the basis of a partial separation of chondroitin sulfate from the car- age of the knee joint of animals injected with sodium sulfate, containing , it is suggested that the major portion, if not all, of the labeled sulfate lfur retained by the articular cartilage may be retained therein as chon- oitin sulfate. Unequivocal proof of this possibility must yet be obtained.

The authors are sincerely grateful to Mrs. E. V. McCollum for making ailable the suckling rats used in the investigation.

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E OXIDATION OF L-TYROSINE BY GUINEA PIG LIVER EXTRACTS

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ith the discovery that a deficiency of ascorbic acid leads to the excre- of partially oxidized tyrosine metabolites, the localization of the nin effect in one or more specific tissues became of first importance in nalysis of the mechanism involved. This objective was achieved when as found that liver slices from deficient animals behaved differently those derived from normal ones. Tyrosine was readily oxidized by nal non-deficient slices but not at all by slices removed from scorbutic ea pigs (1). However, upon addition *in vitro* of the crystalline vitamin deficient slices regained their ability to oxidize the amino acid. As a equence of these findings, it was decided to study the rôle of the vita- in cell-free preparations which, as is well known, lend themselves more factorily to the chemical characterization of the enzymes involved. en cell suspensions made from guinea pig livers had been previously by Bernheim and Bernheim (2), who reported that such preparations zed tyrosine with an uptake of extra oxygen equivalent to 4 atoms per of amino acid present, as do preparations from several other species. and his associates (3, 4) have also described experiments of a similar with pig liver extracts in which the same ratio has been readily obd. In a later paper Bernheim (5) found that guinea pig liver homotes oxidized "tyrosine too slowly to obtain definite end-points." rtunately, the comparison of the normal and scorbutic state in a prob- f this type requires the use of the guinea pig.

erefore, preparations were made from normal guinea pigs according e procedure of the above authors. With tyrosine as the substrate ppreciable rate of oxidation was observed but the variability of total tion and the failure to obtain the expected ratio of 4 indicated diffi- s which would not immediately permit precise comparison of normal corbutic livers. The oxidative behavior of cell-free suspensions and cts from normal liver was studied in detail, the results obtained and er properties relative to tyrosine oxidation being herewith described.

EXPERIMENTAL

ung guinea pigs were maintained on a diet of Purina rabbit chow plete ration) supplemented with either green food or crystalline ascorbic acid. For each experiment the liver was removed from the stun and decapitated animal after thorough bleeding. According to the p cedure of Bernheim and Bernheim (2) it was chopped and ground i mortar with sand. "For every gm. of tissue 0.5 to 1.0 cc. of 0.05 M ph phate buffer, pH 7.8, was added and the suspension pressed throu muslin." In our case, disodium phosphate and monopotassium phosph were used in making the buffer and we also adjusted the suspension to p 7.8 before use. 1 ml. was used in the main compartment of each fla and either 0.5 mg. (2.76 micromoles) or 1.0 mg., unless otherwise indicat of L-tyrosine in 1 ml. of buffer was placed in the side arm. In suital

TABLE I

Tyrosine Oxidation by Guinea Pig Liver Homogenate

Homogenate was prepared by the method of Bernheim. Each ml. was equival to 0.5 gm. of liver. 1 mg. of tyrosine contained in 1 ml. of 0.05 M buffer was us The incubation time was 3 hours.

pH	Oxygen consumption		
	Control values	Excess oxygen	Oxygen / Tyrosine
	microliters	*microliters*	*atoms per mole*
7.8	368	111	1.80
	413	115	1.86
	270	192	3.11
	340	108	1.74
Average			2.13
7.4	380	156	2.53
	386	169	2.74
	324	94	1.52
Average			2.36

controls, tyrosine was omitted from the buffer contained in the side ar Carbon dioxide was absorbed in the usual fashion. Incubation w allowed to proceed for 3 hours, or for other times as stated, and at 37.5 Oxygen uptake was measured at intervals and the control values su tracted from the experimental values in order to determine the substra or excess oxygen.

In early experiments, illustrated in Table I, wide variation of the oxyg consumption of the controls, of the substrate oxygen, and the calculat oxygen to tyrosine ratio was observed. Furthermore, the ratio failed approach the expected value of 4. The excess oxygen was in all cas more than 25 per cent of the control values but yielded an average oxyg to tyrosine ratio of 2.13 atoms per mole of tyrosine present. In adjusti ne pH of these homogenates, it was found that during the grinding process alues of 6.9 to 7.4 were obtained. Consequently, pH 7.4 was used in lditional experiments. As may be seen in the last three experiments of able I, no great improvement was achieved and the average ratio of 2.36 as obtained.

The same technique of preparing liver tissue was used in many additional xperiments. However, the all-glass homogenizer offered greater conenience and freedom from contamination and was consequently used xtensively. By centrifuging either type of preparation a suspension, eadily pipetted and cell-free, was obtained. No real difference between he two types of extract in either basal or substrate oxygen was observed.

The failure to obtain maximum oxygen uptake is understandable, for Bernheim (2) has pointed out that it is achieved "provided the tissue preparation is sufficiently concentrated." Obviously in these experiments the atio of enzyme to substrate concentration was inadequate. In order to letermine the ideal ratio for guinea pig liver additional experiments with ncreased tissue and decreased tyrosine quantities were carried out. For he sake of comparison an assumption not entirely true was made. For xample, when 1 ml. of buffer was added to 1 gm. of liver, it was assumed hat 1 ml. of the resulting preparation represented 0.5 gm. of liver. However, on this basis experiments at different levels have been compared. n Fig. 1 the excess oxygen to tyrosine ratio at 3 hours is plotted against he gm. of liver per mg. of tyrosine. Approximately equal numbers of alues at pH 7.4 and 7.8 are represented. It is to be observed that a ypical enzyme concentration curve is obtained and that with the higher atio of tissue to substrate the oxygen to tyrosine ratio of 4 almost results. This latter was accomplished with 0.5 gm. of liver and 0.25 mg. of tyrosine n the reaction vessel. Thus it may be concluded that normal guinea pig ver homogenates likewise oxidize tyrosine with an uptake of 4 atoms of xygen.

Added evidence for the conclusion that substrate oxidation in a given eriod is dependent upon the ratio of tissue to tyrosine may be obtained n another way. Incubation of different amounts of tyrosine with 1 ml. uantities of the same liver preparation yields the typical substrate conentration curve. In one such experiment, the same amount of extract 1 ml., equivalent to 0.5 gm. of liver) with 0.25, 0.5, and 1.0 mg. of tyrosine ielded oxygen to tyrosine ratios of 3.49, 2.86, and 2.68 respectively. The arger oxygen values in microliters are obtained with the larger quantities f tyrosine, although the greater oxygen to tyrosine ratios are obtained with he smaller substrate quantity. In no case has there been encountered a rue inhibition due to excess substrate, as Bernheim and Bernheim have uggested (2).

Further confirmation may be seen in Table II in which a dilution-t
experiment is summarized. In this the liver was ground with 1 ml.
buffer per gm. according to the Bernheim technique. 0.4, 0.6, 0.8, and

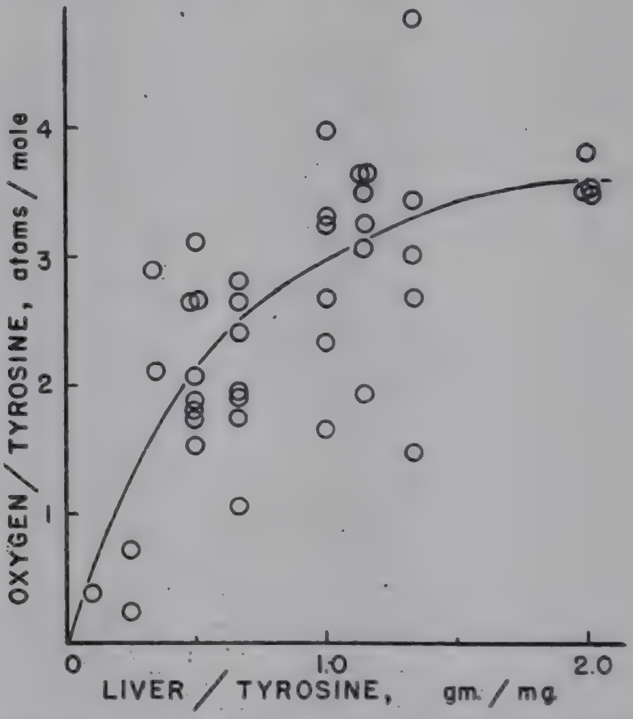

FIG. 1. Tyrosine oxidation at different ratios of liver to tyrosine. Each cir
indicates the oxidation of the tyrosine present at the corresponding ratio of liver
substrate. The forty-one points were obtained with thirty-five guinea pigs. T
smooth curve corresponds approximately to the average values at the different rati

TABLE II

Tyrosine Oxidation by Liver Homogenate

Homogenate was prepared according to the method of Bernheim and adjusted
pH 7.4. 0.25 mg. (1.38 micromoles) of tyrosine was employed. The reaction volu
in each flask was 2.0 ml. and the incubation time was 2 hours.

Liver	Oxygen		Oxygen / Tyrosine	Tyrosine oxyg
	Control	Experimental		
gm.	*microliters*	*microliters*	*atoms per mole*	*microliters per g liver*
0.2	86	97	0.71	55
0.3	125	152	1.79	90
0.4	192	228	2.33	90
0.5	257	305	3.11	96

ml. of the resulting extract were placed in respective pairs of flasks, bu
being added where necessary to make 1 ml. The final reaction volume
2.0 ml. in all the flasks, the experimental ones containing 0.25 mg. of ty

ne. With increasing quantities of tissue the control oxygen values at 2 urs increase, as is to be expected. Likewise, the experimental values ow the same increase, and the calculated oxygen to tyrosine ratios likeise increase. The substrate oxidation per gm. of tissue is remarkably nstant, except with the least amount of liver, as may be seen in the last lumn of Table II. This one discrepancy emphasizes the critical nature tissue concentration in avoiding limiting concentrations of necessary ctors in the enzyme system.

Various modifications of the method of conducting the experiments have een studied, but in no case has there been a significant improvement in the nzyme activity. However, in keeping with the suggestion of Felix (3, 4) e buffer concentration in subsequent experiments has been 0.2 M in rder to stabilize the system more completely. Particularly is this necesary when larger quantities of tissue are used. Simultaneously with the udy of possible modifications other characteristics of the enzyme system ave been determined.

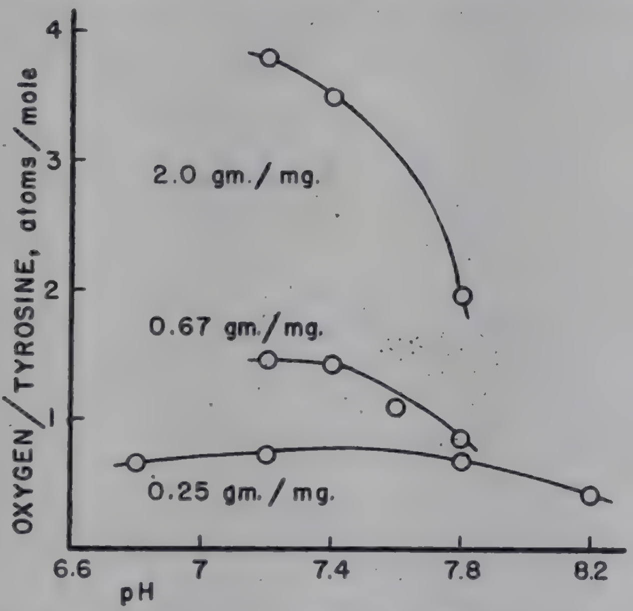

FIG. 2. The effect of pH on tyrosine oxidation illustrated with the results of three ifferent experiments. Each curve was obtained with the ratio of liver to tyrosine dicated by the figures accompanying the curve.

In the first place, the pH of the over-all reaction has been investigated. he findings are summarized in Fig. 2, from which it may be seen that the ptimal value is in the neighborhood of pH 7.2 to 7.4. This finding may e contrasted with that of Felix and his associates (3), for they found that milar preparations from pig liver exhibited optimal activity at pH 7.8. urthermore, they reported definite separation of the steps of the reaction y choice of the pH. With guinea pig liver such a separation has not been ncountered.

One of the most interesting characteristics of the homogenates is th stability upon storage in the cold. An extract was prepared in the us fashion and a portion tested immediately. The remainder was stored 0–4° and small portions retested, after readjustment of pH, at the ti intervals indicated in Table III. As would be expected, the cont oxygen consumption for 3 hours of incubation decreased very rapidly duri 24 hours of storage. In the same length of time no decrease in the abili of the preparations to oxidize tyrosine was observed. Even with 48 hou of storage no significant part of the original activity had disappeare With the second preparation 87 per cent remained after 4 days of stora and with the first preparation a definitely measurable quantity (18 p cent) still remained at 6 days. Consequently it may be concluded that the form of concentrated liver homogenates the tyrosine-oxidizing enzy system is remarkably stable. This in turn suggests that the more unstab types of enzymes are not components. For example, it would seem somewhat unlikely that the cytochrome system is involved.

TABLE III

Stability of Tyrosine-Oxidizing System

Storage time	Experiment I		Experiment II	
	Control oxygen	$\frac{\text{Oxygen}}{\text{Tyrosine}}$	Control oxygen	$\frac{\text{Oxygen}}{\text{Tyrosine}}$
hrs.	*microliters*	*atoms per mole*	*microliters*	*atoms per mole*
0	406	2.61	495	2.19
3	231	3.07		
24	219	2.97	240	2.16
48	147	2.87	205	2.01
72			157	1.89
96			114	1.91
145	157	0.49		

Of importance in the further fractionation is the question of the solubility of the enzyme system in the extracting buffer. Even the centrifuge homogenates upon microscopic examination showed the presence of su pended small particles, including clumped nuclei and other types of ce débris. By comparing the activity of an ordinary homogenate with fraction of the same homogenate that had been subjected to centrifugin at 18,000 R.P.M., evidence for the solubility of the system was obtaine After centrifuging for a total of 30 minutes, the contents of the centrifu tube were separated into three fractions. The upper third was qui watery, although colored, while the middle third was somewhat more visco and still contained finely divided particles in suspension. The botto action consisted of fairly well packed solid material which was resuspended in an equivalent volume of buffer. The oxygen consumption of the gular preparation and of each fraction was determined, with the results hown in Fig. 3. It is obvious that the supernatant liquid retained the bility to oxidize tyrosine. While differences in the uptake of oxygen in he control flasks occur to some extent, the oxygen to tyrosine ratios shown ith the first three pairs of curves represent after 5 hours 79.5, 75.9, and 8.5 per cent of the expected ratio of 4 for the untreated preparation and pper layer and middle layers, respectively. In contrast the substrate xygen of the heaviest fraction is only 12.5 per cent of the maximum value.

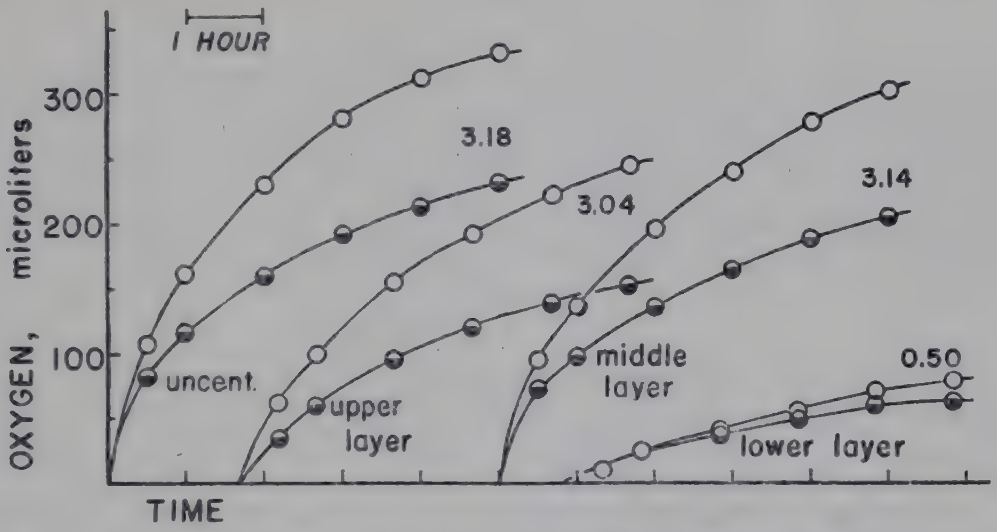

Fig. 3. The oxygen consumption in control and experimental flasks obtained with the usual homogenate and the same homogenate centrifuged at 18,000 R.P.M. ○, homogenate plus tyrosine; ◒, homogenate only. The first pair of curves corresponds to the original preparation. The second, third, and fourth pairs of curves represent the upper, middle, and lower layers, respectively, of the centrifuged material. The values accompanying each pair of curves indicate the ratio of oxygen to tyrosine present (atoms per mole) in each case.

The liquid layers thus account for nearly all of the activity of the original preparation.

With this demonstration of the solubility of the enzymes it then became possible to undertake the study of their composition. Their behavior upon dialysis was ascertained in order to approach their fractionation more intelligently. Centrifuged homogenate was dialyzed in the cold against 0.2 M phosphate at the pH shown in Table IV. For comparison the activity of the undialyzed preparation, measured as soon as it was prepared, s shown. From the results of the first five experiments, it is apparent that at either pH 7.4 or 6.0 dialysis for 20 or more hours leads to a complete loss of activity. That this cannot be due to storage has been shown by those experiments above which established the remarkable stability of the enzymes. With shorter dialysis the loss of activity may be followed. F example, at pH 6, 49 per cent of the original activity is lost in 2 hours, ar in 4 hours only 5 per cent remains. From these experiments it may concluded that dialyzable factors are necessary to the uptake of even t first atom of oxygen in tyrosine oxidation. Further, these factors ar removed from the protein portion very rapidly on dialysis.

As evidence that the protein remains active during the dialysis the resul obtained with addition of boiled extract to completely inactive dialyze extracts may be cited. The boiled extract represented the water-solub

Table IV

Dialysis of Liver Extracts

Freshly prepared extracts at the concentration indicated were dialyzed in the co against 0.2 M phosphate with frequent changes of dialyzing medium. The pH valu at which the dialysis was conducted are shown. In testing, the pH was 7.4, the ori inal test being made as soon as the extract was prepared. The substrate was 0.5 m of tyrosine and the reaction volume varied from 2.0 to 2.2 ml., 1 ml. of extract bei used. The oxygen values in all cases are the totals obtained at 3 hours.

Guinea pig No.	Liver	Oxygen consumption					
		Undialyzed		Dialyzed			
		Control	$\frac{\text{Oxygen}}{\text{Tyrosine}}$	pH	Time	Control	$\frac{\text{Oxygen}}{\text{Tyrosine}}$
	gm. per ml.	*microliters*	*atoms per mole*		*hrs.*	*microliters*	*atoms per mol*
415	0.34	225	1.00	7.4	20	29	0
425	0.5	194	3.15	7.4	27	71	0
426	0.5	255	2.36	7.4	29	57	0
439	0.34	168	1.55	6.0	22	51	0
450	0.34	136	1.52	6.0	22	59	0
440	0.5	180	3.37	6.0	2	114	1.72
					4	75	0.17
					8	62	0

portion obtained from the usual homogenate by heating it in a boiling wate bath. This boiled extract consumed oxygen in insignificant quantitie when tested alone or with tyrosine. Examination of the values of Tabl V, particularly those of the last column, discloses significant tyrosine oxidation upon combining the extract and the dialyzed preparation, even thoug the original activity has not been regained, except in one case. The origina activity for all but two experiments is recorded in Table IV, correspondin preparations being indicated by the number of the guinea pig. From th it may be concluded that dialysis removes components necessary to tyrosir oxidation and consequently the protein fraction becomes enzymaticall inactive. These components may be replaced with a boiled extract of liv ssue with a subsequent return of activity. Thus, the protein is reasonably able to the conditions existing during dialysis as well as storage, and, even ore important, the dialyzable or non-protein components are thermo-able under the conditions employed. More recently it has been demon-rated that even better reactivation occurs when a concentrated dialysate employed instead of the boiled extract.

One additional feature of the activity of the liver preparations requires xamination in order to facilitate fractionation of the system and, for that atter, comparison of the normal and scorbutic animals. The fact that

Table V

Reactivation of Dialyzed Homogenates by Boiled Liver Extracts

The boiled extracts were prepared by heating homogenates at the concentration nd pH shown for 10 to 30 minutes in a boiling water bath. The centrifugates were sed in the quantity indicated, the equivalents indicating the proportion of liver of oiled extract to liver of dialyzed extracts. The tests were carried out as in Table V, except that the reaction volumes were 2.5 to 2.7 ml. The guinea pig numbers epresent the source of dialyzed preparation as described, with one exception, in able IV.

Guinea pig No.	Boiled extract				Oxygen consumption		
	Liver	pH	Amount in test		Control	Experimental	Oxygen / Tyrosine
	gm. per ml.		*ml.*	*equivalents*	*microliters*	*microliters*	*atoms per mole*
415	0.67	7.0	0.5	1	121	165	1.42
415*			0.5	1	117	149	1.00
426	0.67	6.0	0.1	0.14	71	85	0.42
439	0.67	7.1	0.5	1	200	244	1.40
439†			0.5	1	162	199	1.18
450	0.67	6.9	0.5	1	127	159	1.03
440	0.5	7.0	0.5	0.5	104	124	0.65
453	0.67	7.0	0.5	1	146	161	0.49

* Retested after 24 hours storage.

† Retested after 72 hours storage.

yrosine oxidation occurs with the uptake of 4 atoms of oxygen and is not eadily separable into individual steps requires the use of as many methods s possible in order to evaluate the reaction mechanism. Consequently, addition to oxygen consumption, colorimetric analysis of the tyrosine emaining at the end of the incubation has been carried out. This is not ntirely satisfactory, since Felix (3) has shown that the first product of e oxidation also reacts in the Millon procedure.

As rapidly as possible after the last manometer readings were taken, the ontents of the flasks were transferred into 1 ml. of 10 per cent metaphos-horic acid contained in graduated centrifuge tubes. Trichloroacetic acid could not be used, since precipitates formed with it in several of the analy cal methods employed. After dilution to 10 ml. and thorough mixing, t solutions were allowed to stand for 30 minutes before centrifuging. A propriate aliquots of the supernatants were then analyzed by means of t Bernhart method (6). The values for the control flasks, which were usua small, were deducted from the experimental values. The difference repr sented the total "tyrosyl" value and included the quantity of added tyr

Table VI

Colorimetric Analysis of Tyrosine Oxidation

Incubations conducted in the usual fashion at pH 7.4 for 3 hours and in 2 ml. total reaction volume. The term, O:T_p, is the ratio of substrate or excess oxygen the tyrosine present at the beginning of the incubation. "Tyrosine oxidized" repr sents the difference between the phenolic value after incubation and the tyrosi originally present, but does not represent added amino acid. The term, O:T_c, the ratio of excess oxygen to "tyrosine oxidized." For fuller explanation the te should be consulted.

Liver / Tyrosine	Oxygen excess	O:T_p	"Tyrosine oxidized"	O:T_c
1.0 mg. tyrosine 5.52 μM				
gm. per mg.	*microatoms*	*atoms per mole*	*micromoles*	*atoms per mole*
0.1	2.12	0.385	1.27	1.67
0.25	3.75	0.679	1.01	3.72
0.25	1.39	0.252	0.60	2.32
0.35	11.7	2.12	2.45	4.77
0.5	11.5	2.08	3.58	3.21
0.67	10.7	1.94	4.05	2.64
0.67	15.4	2.78	4.47	3.45
0.67	15.5	2.81	4.57	3.38
0.5 mg. of tyrosine (2.76 μM)				
1.14	6.25	2.26	0.95	6.58
1.14	9.02	3.26	1.75	5.15
Average				3.68

sine and first product remaining at the conclusion of the incubation. B subtracting this quantity from the original tyrosine, a value designated the "tyrosine oxidized" has been obtained. From this it then has bee possible to calculate an additional term, the ratio of substrate oxygen "tyrosine oxidized," which is designated as O:T_c. It must of course distinguished from the original ratio of oxygen to tyrosine present at th beginning of the experiment (O:T_p). The results of a series of experimen in which the method was employed are recorded in Table VI. With increa

; amounts of liver per mg. of tyrosine, the excess oxygen and oxygen to osine present increase as shown in the above paragraphs. Likewise, may be seen, the "tyrosine oxidized" as measured by the colorimetric ethod also increases. At the same time, the ratio of oxygen to tyrosine alculated" approaches the theoretical value of 4. This value, of course, n be achieved only when all of the tyrosine which undergoes the first ige of oxidation proceeds on through to complete oxidation without cumulation of residual chromogenic first product. Further, when both e $O:T_p$ and $O:T_c$ equal 4.0, the reaction may be regarded as having ached the stage of completion previously ascribed to liver preparations. ıe three out of the ten values in Table VI which are greater than 4.0 are obably the result of errors inherent in the experiments and which sumate in the ratio, $O:T_c$. In spite of this objection, however, the additional ol makes possible a more complete analysis of the reaction mechanism d the enzymes involved.

Additional analyses have also been made but the results serve only to nfirm the well known fact that tyrosine is ketogenic in its oxidative catabism, and in its conversion to acetone bodies yields no α-keto acid or nmonia in the presence of liver homogenates. Consequently, these sults will not be presented in detail here.

DISCUSSION

Experiments with a large number of guinea pigs have shown that the ers of this species yield a cell-free preparation capable of oxidizing tyroıe to the extent that 4 atoms of oxygen are consumed for each mole of rosine. This oxidation occurs if the concentration of tissue is sufficiently eat. Consequently, the guinea pig, in this respect, is in the same category th other species such as the rat studied by Bernheim and Bernheim (2) d the pig as studied by Felix and his associates (3). As a result of these periments, it becomes possible to understand instances in which the aximum oxygen ratio is not obtained. For example, Edson (7), using t liver homogenate, obtained only 2 atoms of oxygen per mole of tyrosine. our own experience, rat liver preparations with the lower liver concentraons frequently fail to show optimal rates of tyrosine oxidation. With gher concentrations or decreased amounts of tyrosine the expected value readily obtained.

It seems reasonable to assume that the above difficulty is indirectly a sult of one important aspect. In all experiments dealing with this oxidaon system, regardless of species, exceptionally large amounts of tissue are quired. The 0.5 gm. of liver per 2 ml. of reaction volume frequently nployed represents a 25 per cent suspension or extract and is to be conasted to the few mg. used in the great majority of enzyme studies in

carbohydrate metabolism and even in some types of protein or amino a
systems. If we may suppose that essentially optimal conditions are be
used, then it must be concluded that the quantity of the enzymes concer
is relatively small and their respective rates are of lower order. T
represents something of a handicap, for the larger quantities of tissue l
to higher and more variable control values and the greater lack of uniform
in substrate oxidation, whether it be measured manometrically or colo
metrically.

A low order of enzyme concentration or activity such as observed in t
case may indeed constitute a less attractive system for study by the che
ist, but should not be regarded as indicative of lack of physiological imp
tance. This system is present in liver tissue and therefore may be consider
an operating part of the total catabolic metabolism. In fact, when o
considers the ease with which phenylalanine may be converted to tyrosi
in the animal body, it is apparent that a more active destruction of tyrosi
in oxidative degradation would decrease the quantity of the two ami
acids available for protein synthesis and other anabolic functions. At t
same time, operation of the system, even at reduced velocity, must decrea
the quantity of tyrosine available for formation of undesirable produc
such as the pressor amines, tyramine, and 3-hydroxytyramine. With litt
further argument in this vein, it can be concluded that this tyrosine-oxid
tive system plays a significant rôle in the total economy of the animal bod
operating under the influence of numerous physiological factors and co
centration gradients. It therefore is worthy of further characterizatio
both from the *in vitro* and the *in vivo* standpoint.

SUMMARY

Cell-free homogenates prepared from guinea pig livers oxidize L-tyrosi
with an uptake of 4 atoms of oxygen per mole of tyrosine present, provide
an adequate quantity of liver per unit of tyrosine is employed. In the
experiments 2 gm. of liver per mg. of tyrosine were necessary for maximu
oxidation.

The tyrosine-oxidizing system of guinea pig liver exhibits maximu
oxidation at pH 7.2 to 7.4 and a remarkable stability upon storage in t
cold. As much as 87 per cent of the original activity remains after 4 da
of storage.

The activity is soluble in the aqueous buffer solution employed rath
than a part of the particulate matter separated by high speed centrifugin

Upon dialysis the preparations rapidly lose their ability to oxidize tyr
sine, but regain it with addition of boiled extract, from which it may b
concluded that thermostable dialyzable components constitute a portion
the enzyme system.

When insufficient enzyme is present to cause complete oxidation, the rosine remaining may be determined by colorimetric means. By comring substrate oxygen and the tyrosine disappearing, a ratio of 4 atoms oxygen per mole of tyrosine oxidized is obtained within the limits of error the methods employed.

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EXCRETION OF ESSENTIAL AMINO ACIDS BY MEN ON A CONTROLLED PROTEIN INTAKE*

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A nutritional project at the Elgin State Hospital (1) provided an opportunity for the study of the excretion of amino acids by a group of twenty men on a controlled protein intake.[1] Data obtained from such a study might be expected to throw some light on amino acid metabolism and might possibly have some clinical significance. The subjects taking part in this study were a group of male mental patients, with varied and unrelated psychiatric diagnoses, who had been on the controlled diet for a period of 3 years, during which time they had maintained their body weight and nitrogen balance. Frequent analyses of the diet indicated its constant composition with respect to nitrogen and B complex vitamins.

Most of the subjects at some time during the period preceding this study had been on a diet which was restricted in thiamine and riboflavin. However, the protein content did not vary, either during the period of restriction or during the subsequent period of supplementation. At the time when the urines were collected for the present study, all the subjects had been on a vitamin-supplemented régime (1) for from 3 to 11 months and were free from any signs of nutritional deficiency.

The protein intake during the 3 years preceding the evaluation of amino acid excretion was approximately 55 gm. per man per day. The average caloric intake per day was 2200 calories. Actual food consumption and food rejection were controlled in a diet kitchen especially equipped for this purpose. Approximately half of the protein in the diet was derived from meat.

Preliminary microbiological assays for urinary amino acids with *Streptococcus faecalis* R indicated that the amount of "free" amino acid in the urine was in most cases small, and that some urines contained a sub-

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[1] A comprehensive description of the dietary phases of this work is given in Bulletin 116 of the National Research Council entitled "Investigations of human requirements of B-complex vitamins."

stance which inhibited the growth of the microorganism. With the idea that the inhibiting substance might be urea some of the urines were heated with hydrochloric acid to hydrolyze the urea. Practically all signs of inhibition of bacterial growth were absent when the hydrolyzed urine was assayed. The fact that the amino acid content of the urine increased several fold as a result of acid treatment indicated that the assays of the hydrolyzed urines would probably be worth while. Accordingly most of the data to be reported were obtained from analyses of urines which had been heated with 6 N hydrochloric acid.

EXPERIMENTAL

24 hour urine samples were collected under toluene. Preliminary tests had shown that when urines were adjusted to pH 6.9 with sodium hydroxide and autoclaved the pH increased to between 8.0 and 8.5 and was occasionally even higher, presumably owing to a change of bicarbonate to carbonate. Because large amounts of urine were required for assay of most of the "free" amino acids, the addition of these urines, varying both in volume and pH, would produce variations in hydrogen ion concentration in the assay tubes. Such a condition would invalidate the results of the assay. To prevent this variable increase in alkalinity on autoclaving, aliquots were acidified immediately after collection with acetic acid to pH 4 and then autoclaved for 10 minutes at 15 pounds pressure. After cooling, the acidity was adjusted to pH 6.9 and distilled water was added to replace the amount evaporated. The urines were then autoclaved for 15 minutes at 15 pounds pressure and stored under sterile conditions in the refrigerator until used for assay. Urines so prepared were used for the assay of "free" amino acids.

To obtain data on the "total" amino acid content, the urines were hydrolyzed as follows: 200 to 300 ml. aliquots of urine prepared for assay of "free" amino acids, as described above, were filtered into tared flasks and evaporated *in vacuo* to a residue of 25 to 30 gm., 1 ml. of 12 N hydrochloric acid was added for each gm. of residue, and the mixture refluxed for 8 hours. The hydrochloric acid was then removed by evaporation to dryness *in vacuo*. The residue was washed onto a filter by means of a stream of hot water and then washed repeatedly with hot water. The filtrate after adjustment to pH 6.9 was diluted to one-half the volume of the urine taken for hydrolysis and then autoclaved for 15 minutes at 15 pounds pressure and stored under sterile conditions in the refrigerator until used for assay.

The amino acid intake was determined from analysis of three dried, 24 hour food composites corresponding to the 3 days urine collection.

Aliquots were hydrolyzed by refluxing for 9 hours with 6 N HCl. After hydrolysis they were filtered, diluted to definite volume, and stored in the refrigerator under toluene until used for assay.

All assays of food and urine were done microbiologically. *Streptococcus faecalis* R was used for the assay of all the amino acids to be reported

TABLE I

Composition of Basal Media

Constituent	Amount per liter of basal medium, final volume	
	Medium A*	Medium B†
	mg.	*mg.*
DL-Alanine	100	100
L-Asparagine	200	200
L-Aspartic acid	200	200
L-Arginine·HCl	50	100
L-Cystine	200	200
L-Glutamic acid	400	400
Glycine	75	75
L-Histidine·HCl·H_2O	50	100
DL-Isoleucine	200	200
DL-Leucine	200	200
DL-Lysine	330	200
DL-Methionine	100	100
DL-Norleucine	100	100
DL-Norvaline	100	100
DL-Phenylalanine	100	
DL-Serine	70	170
DL-Threonine	170	170
DL-Tryptophan	100	100
L-Tyrosine	100	100
DL-Valine	200	200
Adenine	10	10
Guanine	10	10
Uracil	10	10
Xanthine	10	10

Constituent	Amount per liter of basal medium, final volume	
	Medium A*	Medium B†
	mg.	*mg.*
Sodium acetate, anhydrous	6,000	6,000
Sodium citrate	20,000	20,000
NaCl	10	10
$MgSO_4 \cdot 7H_2O$	200	200
$FeSO_4 \cdot 7H_2O$	10	10
$MnSO_4 \cdot 4H_2O$	10	10
K_2HPO_4	5,000	500
KH_2PO_4		500
NH_4Cl	170	170
Glucose	20,000	20,000
	γ	γ
Thiamine	500	500
Riboflavin	500	500
Nicotinic acid	1,000	1,000
Calcium *dl*-pantothenate	500	500
Pyridoxamine·2HCl	300	300
p-Aminobenzoic acid	300	300
Biotin	2	2
Folic acid‡	17	17

* For assay of arginine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, and valine, the amino acid to be estimated being omitted from the medium.

† For assay of phenylalanine.

‡ Lederle synthetic.

here with the exception of phenylalanine for which *Lactobacillus delbrueckii* LD5 was used.

The contents of the basal media are listed in Table I. Medium A is based on reports by Greenhut, Schweigert, and Elvehjem (2) and of

Baumgarten, Mather, and Stone (3). Medium B is with some modifications that described by Stokes, Gunness, Dwyer, and Caswell (4). Stab stock culture and inoculum for assay were prepared according to the method of Stokes and Gunness (5) with the exception that 1 gm. of yeast extract was added to the ingredients listed by the above authors. In the case of assays with *Streptococcus faecalis* R the extent of bacterial growth was determined turbidimetrically, after 20 to 24 hours incubation at 35° in a forced draft oven. A Coleman spectrophotometer with wave-length set at 650 mμ was used. Variable turbidities which might be present in the different urines were eliminated by centrifuging and using only the clear supernatant liquid for assay. To eliminate effects of turbidities resulting from the reaction of the urine with the basal medium or light absorption by the urine, blanks were run on all urines. The blank tubes were treated exactly as the others with the exception that they were not inoculated. In the case of phenylalanine assays with *Lactobacillus delbrueckii* LD5, measurements were made by titration of the acid produced after 72 hours incubation at 37°. Table II shows the results obtained from the analyses of the urines for "total" amino acids and compares the amino acids excreted with the amounts ingested. Table III gives a similar tabulation of the "free" amino acids.

TABLE II

"Total" Essential Amino Acids in Urine of Twenty Subjects on Controlled Diets

Amino acid	No. of days	Average daily diet	Average excretion per day ± standard deviation	$\frac{\text{Excreted}}{\text{Ingested}} \times 100 \pm$ standard deviation
		gm.	mg.	
Arginine	3	3.0	18.2 ± 3.1	0.62 ± 0.13
Histidine	3	1.9	66.9 ± 26	3.5 ± 1.3
Isoleucine	3	3.1	10.0 ± 2.9	0.33 ± 0.09
Leucine	2	4.5	15.5 ± 4.2	0.33 ± 0.10
Lysine	2	3.1	16.8 ± 6.6	0.56 ± 0.22
Methionine	3	1.2	6.2 ± 1.4	0.54 ± 0.11
Phenylalanine	3	2.9	13.0 ± 3.4	0.47 ± 0.14
Threonine	3	2.6	20.2 ± 7.4	0.78 ± 0.27
Valine	3	3.0	19.0 ± 5.4	0.62 ± 0.17

The treatment of the urine described above, *i.e.* heating the urine at pH 4 followed by neutralization and again heating, and the filtration of the aliquots of such urines before their hydrolysis make it unlikely that more than traces of coagulable protein were present in the urines. The several fold increase in amino acid content after hydrolysis must then be due to splitting of non-coagulable amino acid complexes. An attempt vas made to obtain an estimation of the larger non-coagulable amino acid complexes by treating a sample of each urine before hydrolysis with Exton's reagent (20 gm. of $Na_2SO_4 \cdot 10H_2O$ and 5 gm. of sulfosalicylic acid in 100 ml.). The amount precipitated was compared turbidimetrically with similarly treated dilute solutions of human serum albumin. Although it is recognized that this affords only a rough approximation, t does give comparative results, which indicate that the amounts of non-coagulable amino acid complexes precipitated by Exton's reagent are a relatively minor part of the "total" amino acid figure obtained.

TABLE III

"Free" Essential Amino Acids in Urine of Subjects on Controlled Protein Intake

Amino acid	No. of subjects	No. of days	Average daily diet	Average excretion per day ± standard deviation	$\frac{\text{Excreted}}{\text{Ingested}} \times 100 \pm$ standard deviation
			gm.	*mg.*	
Arginine	6	1	3.0	4.1 ± 1.9	0.14 ± 0.07
Histidine	19	1	1.9	38.0 ± 15.1	2.17 ± 0.98
Isoleucine	6	1	3.1	0.9 ± 0.4	0.04 ± 0.01
Leucine	6	1	4.5	1.0 ± 0.4	0.02 ± 0.01
Lysine	5	1	3.1	5.0 ± 1.0	0.17 ± 0.04
Methionine	19	1	1.2	3.0 ± 0.9	0.27 ± 0.06
Phenylalanine	6	1	2.9	4.7 ± 1.5	0.20 ± 0.05
Threonine	9	1	2.6	4.3 ± 1.7	0.17 ± 0.07
Tryptophan	20	2	0.52	11.6 ± 4.1	2.4 ± 1.1
Valine	6	1	3.0	2.4 ± 0.6	0.08 ± 0.02

RESULTS AND DISCUSSION

The data for the excretion of "total" amino acids given in Table II how that the values range from 6.2 mg. per day in the case of methionine o 66.9 mg. per day in the case of histidine. The values are much lower han those reported by workers using chemical methods (6–10). Others vho have used microbiological methods (11–15) have also obtained reults lower than those obtained by chemical procedures. To account or these differences, certain possibilities have been suggested. Part of he amino acids may be excreted in the form of the unnatural isomer, vhich cannot be utilized by the assay organism, but would take part in he chemical reaction. So far, however, it has not been demonstrated hat natural amino acids are converted by the body into their optical ntipodes. It is also possible that certain amino acid metabolites or ther urinary constituents may take part in the chemical reaction without being capable of utilization by the microorganism. Finally, there emains the possibility that some urinary constituents may inhibit or accelerate growth of the organism.[2] Sauberlich and Baumann (12) in di cussing this point state, "It is doubtful whether the method for a sing amino acid has been studied adequately for the influence of compound other than the one being determined, and it might be questioned wheth absolute value should be ascribed to the results of microbiological assay on so complex a mixture as urine. On the other hand, the parallelis observed in the present study between the amounts of protein ingeste and the amounts of amino acids excreted in microbiologically available form suggests that the assays yield results that are at least roughly quantitative."

In the present study recoveries of amino acids added to hydrolyze urine ranged from 88 to 110 per cent. Only an occasional hydrolyze urine exhibited drift in one of the amino acid assays; *i.e.*, the results wer not proportional to the different amounts of urine added. There wer no cases of drift caused by any of the 59 urine samples in the analyse for arginine, histidine, phenylalanine; for leucine, none out of 39; for isoleucine and methionine, 1 each out of 59; for valine, 2 out of 59; for threonine, 3 out of 59; and for lysine, 3 out of 38 urines. The fact that tw urine samples exhibited drift in the assay of only two of the nine amin acids and six other urines exhibited drift in the assay of only one of th nine acids would seem to indicate that there was an interference in th utilization of particular amino acids when present in small quantitie rather than a more general growth inhibition of the microorganism.

The amounts of "total" amino acids found to be excreted in the urin in the present study are less than those recently reported by other worker (13, 15, 16) who have studied the content of amino acids in hydrolyze urines, using microbiological methods. Table IV compares the sets c results obtained by different laboratories. Differences may in part b due to differences in amino acid intake. The last three columns of Tabl IV give values for the daily amino acid requirements as quoted by Bloc and Bolling (17). Comparison of the suggested requirements with th amounts actually consumed indicates the improbability that very muc of any amino acid was consumed in excess of the daily requirement. O the other hand, since body weight was maintained for over 3 years on thi diet, it is apparent that there was no amino acid deficiency. Under thes conditions one would expect the excretion in the present study to be clos to minimum values, provided that excretion is dependent upon the amoun ingested. That such a relationship exists is indicated in Table IV b comparing the results obtained by Dunn *et al.* on subjects on norma iets and on K ration, and by comparing the results obtained by Scheffner t al. (16) on two subjects with slightly different intakes of amino acids. With the exception of histidine, lysine, and threonine, our results agree with those of Scheffner *et al.*, particularly in the case of Subject 1 of the atter authors, whose amino acid intake was very close to that of our subjects.

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[2] Dunn *et al.* (13) have reported that a worker in their laboratory has found tha hippuric acid has a greater growth-promoting activity per mole than does glyci for *Leuconostoc mesenteroids* P-60.

Sauberlich and Baumann (12) have found that the amount of amino acids excreted by rats and mice is dependent upon the amount in the diet. These authors have also found differences in excretion between he two species. Pearce, Sauberlich, and Baumann (18) have studied he excretion of amino acids by mice fed incomplete proteins and have ound that on such deficiency diets there is an increase in excretion of amino acids other than the one which was absent from the diet.

The failure of the subjects to lose weight on a diet containing only 2200 calories is indicative of their general inactivity. Their sedentary behavior, though punctuated by administrative attempts to supply exercise in the form of walks and recreational activities, did not allow for much muscular metabolism. Perhaps this lack of muscular activity is n part responsible for the small excretions obtained.

Table III summarizes the findings with regard to the excretion of "free" amino acids. The term "free" amino acid indicates those forms of the amino acid available for growth of the assay organism without the intervention of acid hydrolysis. It will include not only the truly free amino acid and its simple salts, but also any combined amino acid (in peptides or other conjugated form) which the organism is able to use for growth. With the exception of tryptophan, the "free" amino acids were not studied as extensively as the "total" amino acids. The tryptophan values of he present study range from 6 to 22 mg. per day, with an average of 11.6 mg. Other values that have been reported are as follows: Schweigert, Sauberlich, and Elvehjem (11) 12 to 30 mg. per day; Frankl and Dunn (19) 13 to 23 mg. per day on normal diets and 28 to 44 mg. on K ration diets; Schweigert, Sauberlich, Elvehjem, and Baumann (20) 6 to 10 mg. per day, with an average of 6.9 mg.

Our values for the excretion of "free" amino acids, with the exceptions noted below, are in fair agreement with those of Steele *et al.* (14). We ound small amounts of leucine, whereas these workers reported that it was absent. Our values for histidine were about one-half and that of hreonine about one-sixth that found by Steele *et al.* Woodson *et al.* (15) report values somewhat larger in almost all cases than those obtained by Steele *et al.* and ourselves.

Individual subjects had a tendency to excrete amino acids at characteristic levels. Six men gave the highest excretion values for each assay,

TABLE IV

Comparison of Urinary "Total" Amino Acids with Ingested Amino Acids

Amino acid	Present study			Dunn *et al.*				Woodson *et al.*	Sheffner *et al.**						Daily requirements as suggested by		
				K ration			Normal diet		Subject 1			Subject 2			Rat growth (Rose)	Amino acid analyses	
	Ingested per day	Excreted per day	Amino acid excreted	Ingested per day	Excreted per day	Amino acid excreted	Excreted per day†	Excreted per day	Ingested per day	Excreted per day	Amino acid excreted	Ingested per day	Excreted per day	Amino acid excreted		Mary	Block
	gm.	*mg.*	*per cent*	*gm.*	*mg.*	*per cent*	*mg.*	*mg.*	*gm.*	*mg.*	*per cent*	*gm.*	*mg.*	*per cent*	*gm.*	*gm.*	*gm.*
Arginine	3.0	18.2	0.61	3.1	41.5	1.31	35.6	23.7	3.18	20.0	0.63	4.67	33.3	0.71	1.2	4.7	4.7
Histidine	1.9	66.9	3.5	2.4	251.6	10.5	188.4	203.3	1.80	120	6.67	2.18	407	18.7	2.4	1.6	2.0
Isoleucine	3.1	10.0	0.32	5.1	19.5	0.39	19.3	20.3	3.60	13.3	0.37	3.80	21.7	0.57	3.0	3.1	3.7
Leucine	4.5	15.5	0.34	7.3	39.0	0.53	31.1	21.2	5.26	16.7	0.32	6.32	35.0	0.55	5.4	9.6	12.6
Lysine	3.1	16.8	0.54	5.4	75.7	1.4	83.0	73.2	3.12	51.7	1.66	4.80	149	3.11	6.0	4.6	5.2
Methionine	1.2	6.2	0.52	2.0	22.0	1.1	11.9	8.6	1.19	5.0	0.42	1.75	11.7	0.67	3.6‡	3.7‡	4.1‡
Phenylalanine	2.9	13.0	0.45	4.0	31.8	0.80	32.6	23.3							4.2	4.2	4.7
Threonine	2.6	20.2	0.78	3.7	66.0	1.78	57.9	53.8	2.50	45.0	1.80	3.24	75.0	2.31	3.6	3.2	3.6
Valine	3.0	19.0	0.63	5.3	36.6	0.69	29.7	19.8	3.55	16.7	0.47	4.10	25.0	0.61	4.2	3.2	3.9

* Averages of two 6 day periods.

† Amounts of ingested amino acid not given.

‡ Values for methionine plus cystine.

e another six men consistently gave the lowest values. There may some correlation between the amount of amino acid excreted and er age or body weight. The older individuals tended to excrete larger unts of amino acids than the younger ones, but the older men aver-l 17 pounds heavier than the younger men. Albanese and Frankston reported that the excretion of tryptophan was proportional to the y weight, but Schweigert, Sauberlich, and Elvehjem (11) found no a correlation.

SUMMARY

he excretion of "total" and "free" essential amino acids has been lied in a group of men on a controlled diet. 55 gm. of protein per were supplied by this diet which permitted the maintenance of body ght and nitrogen balance for a period of over 3 years.

verage excretion of "total" amino acids varied from 6.2 mg. per day he case of methionine to 66.9 mg. per day in the case of histidine. rage excretion of "free" amino acid ranged from 0.9 mg. per day in case of isoleucine to 38 mg. per day in the case of histidine. The ount of "total" amino acid excreted was several fold that of the "free" no acid excretion.

he amounts excreted were lower than some workers have found, g similar microbiological assays. This may be due to the limited ount of essential amino acids ingested by the subjects used in this ort.

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THE METABOLISM OF RADIOACTIVE PENTOBARBITAL IN MICE*

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Plate 1

(Received for publication, December 27, 1948)

The synthesis of radioactive urea (C^{14}) of high specific activity[1] permits e to trace the metabolism of urea and many of its readily synthesized rivatives, *i.e.* the barbiturates.

The universal use of the barbiturates in clinical medicine makes an inestigation of their metabolism in the animal of interest. Pentobarbital dium (nembutal) (tagged with C^{14}, specific activity 8400 counts per cond per mg.) has been prepared in these laboratories from radioactive ea.[1] The metabolism of this compound has been the subject of reeated investigation both *in vivo* and *in vitro*. Recently the problem s been attacked by van Dyke, Scudi, and Tabern (1) who used the N^{15}-beled compound. However, since the compound labeled with C^{14} afrds greater sensitivity and accuracy in the analytical methods, the relts of our investigation are reported here.

EXPERIMENTAL

Each of twelve female mice (CF_1 strain) was injected intraperitoneally ith 0.5 mg. of C^{14}-labeled sodium pentobarbital. The animals in groups three were placed in a series of all-glass metabolism cages (2). No esthetic effect was noted. Urine and exhaled CO_2 were collected at 3, 12, 24, 48, and 72 hours. The urine was analyzed for radioactivity by rect plating (3) and for the CO_2 by preparing $BaCO_3$ plates. Feces were llected over a period of 72 hours and completely extracted of radiotivity in a Soxhlet apparatus with water as the solvent. Animals were crificed at 3 hour intervals for the first 12 hours, the tissues burned, d the resulting CO_2 precipitated as $BaCO_3$ and analyzed for radiotivity.

The metabolic products in the urine were studied by means of the ethods of filter paper chromatography. 0.1 ml. of pooled urine (0 to

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* This document is based on work performed under contract No. W-7405-eng-36 the Atomic Energy Commission.

[1] Murray, A., 3rd, and Ronzio, A., manuscript in preparation.

12 hours) corresponding in radioactivity to 6 γ of original pentoba was placed in a transverse line (in three repeated doses to keep the narrow) on a strip of Whatman No. 1 filter paper 2 × 42 cm.; on ond strip 0.1 ml. of urine plus 0.03 γ of radioactive pentobarbital placed; on a third strip 0.03 γ of radioactive pentobarbital was pl. Chromatograms of groups of three such as that described above sentially, the application of the isotope dilution technique to chr tography) were developed in the following solvent systems, by mea one-dimensional paper chromatography (4): (*a*) hexanol-ethanol (saturated with water, (*b*) *n*-butanol saturated with 3 per cent ammo hydroxide, (*c*) *n*-butanol saturated with water, (*d*) *n*-butanol-eth (9:1) saturated with water, (*e*) *n*-butanol-acetone (3:1) saturated water. Radioautographs of the finished chromatograms were mad placing the filter paper strips in contact with no-screen or Blue B x-ray film in casettes for a 6 day period to permit the β-rays of C expose the film.

RESULTS AND DISCUSSION

78 per cent of the injected activity was recovered in the urine an per cent in the feces, making the total recovery 80 per cent of the injec dose. This low recovery value is presumably due to errors in injec and collection of specimens. The activity found in the feces might h resulted from contamination with the urine. Of the activity found the urine 90 per cent was excreted during the first 12 hours of the exp ment. The radioactivity found in the tissues after 3 hours (the earl specimen obtained) was too small to evaluate quantitatively, but it wo appear that at this time it was not concentrated in any specific tiss No activity was found in the exhaled CO_2. This lends support to results that cleavage of the ring to urea and malonic acid does not occ as it has been previously shown that 20 per cent of radioactive urea jected into mice is converted to CO_2 (5).

Fig. 1 shows a typical analysis of a urine specimen collected fro mouse that had been injected with radioactive pentobarbital. T radioautographs of the chromatograms indicate at least five radioact metabolites in the urine and in addition demonstrate the purity of original injected compound. In agreement with the work of Sho and coworkers (6), who were unable to recover any pentobarbital in urine of dogs, a comparison of chromatograms *A*, *B*, and *C* in the va ous groups shows that certainly less than 0.5 per cent (0.03 γ/6 γ) of pentobarbital, if any, is excreted unchanged. Similar chromatogra with use of radioactive urea and urine demonstrated that less than per cent, if any, of the ring is split directly to urea.

n Dyke and coworkers (1) with the use of N^{15}-labeled pentobarbital . small percentage of N^{15} in the urea fraction (less than 7 per cent, admittedly high error). Assuming their results were correct and ning that the metabolism in mice and dogs is similar, one would ude that the N^{15} appearing in the urea does so by virtue of some formation other than direct splitting of the ring to urea; otherwise ame per cent of C^{14} activity would be found in the form of urea. ; isotope dilution techniques and repeated recrystallization, these ors conclude that 2.7 per cent of the drug in their experiments is ted unchanged. From Fig. 1 it is seen that the major metabolite entobarbital has solubility properties very similar to the original barbital. The likelihood of the occurrence of coprecipitation in ted recrystallization may be significant under these conditions and fore some doubt may be cast on the finding of 2.7 per cent of unged drug (7).

om the position of the metabolities on filter paper strips a method he further investigation and concentration of the metabolic product ntobarbital is shown.

SUMMARY

e metabolism in mice of sodium pentobarbital (C^{14}-labeled) ind intraperitoneally has been investigated. Of the 80 per cent of ctivity recovered, 78 per cent was found in the urine and 2 per cent e feces. No activity appeared in the exhaled CO_2.

ter paper chromatographic analyses of the urine indicate the pres of five radioactive metabolites, none of which are urea or the orig entobarbital.

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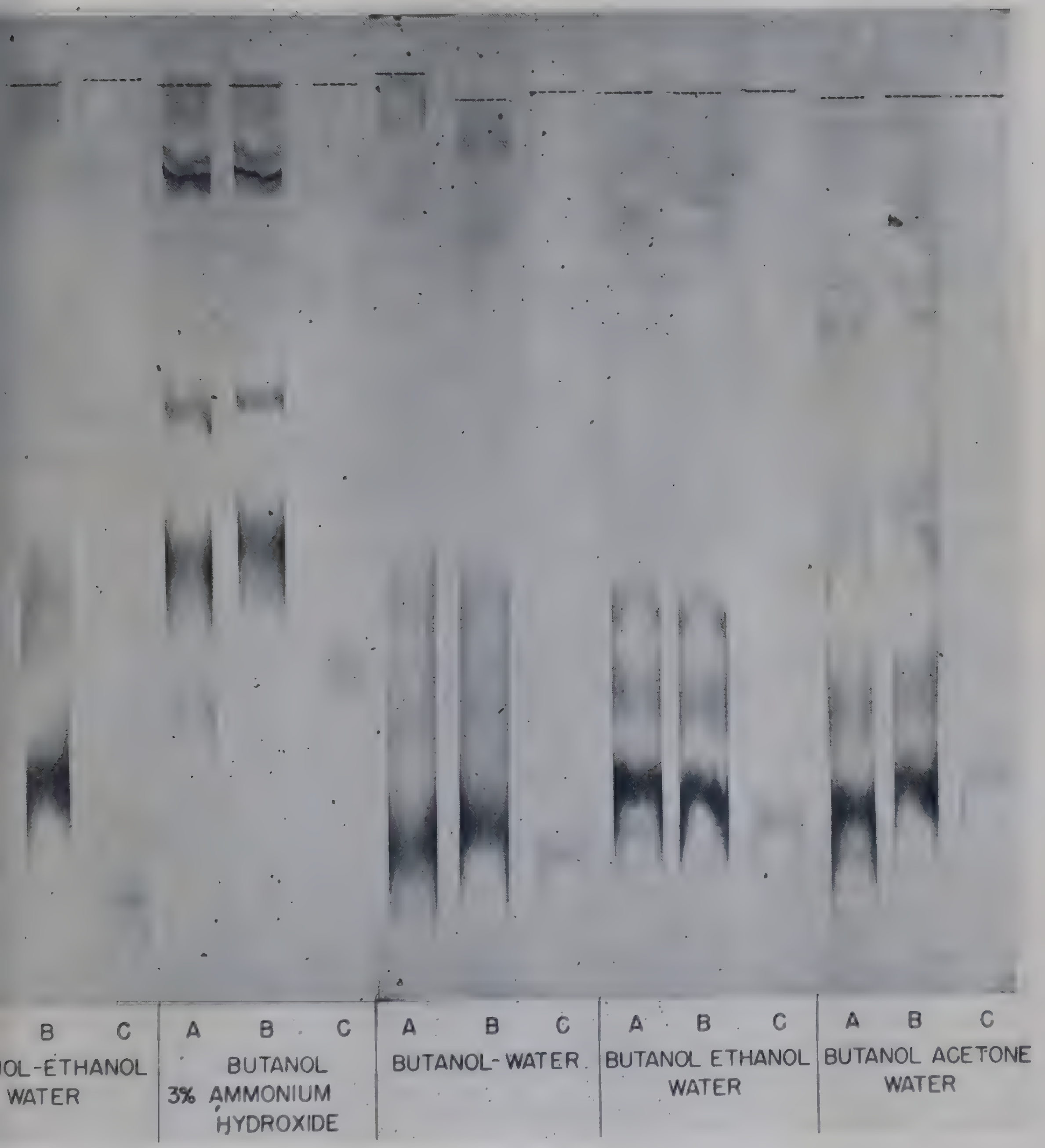
EXPLANATION OF PLATE 1

. 1. A typical analysis of a urine specimen collected from a mouse injected with ctive pentobarbital. 0.01 ml. of test solution was placed on each filter paper dotted line. The filter paper strip is 2 × 42 cm. Whatman No. 1. *A*, urine adioactive nembutal; *B*, urine; *C*, radioactive nembutal.


B C
OL-ETHANOL
WATER
A B C
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3% AMMONIUM
HYDROXIDE
A B C
BUTANOL-WATER
A B C
BUTANOL ETHANOL
WATER
A B C
BUTANOL ACETONE
WATER

(Roth, Leifer, Hogness, and Langham: Metabolism of pentobarbital)

IE EFFECT OF 3-INDOLEACETIC ACID ON THE RESPONSE OF LACTOBACILLUS ARABINOSUS 17-5 TO NICOTINAMIDE

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(Received for publication, November 15, 1948)

Lactobacillus arabinosus 17-5 has been widely used as an assay organism nicotinic acid (NA) since the development of the method by Snell and right (1). Although it has been realized that other substances present in sue extracts may interfere with the bioassay, the nature of such substances s not been elucidated.

During an investigation of tryptophan metabolism in the pea plant, we idied the possible conversion of this compound to nicotinic acid, since such transformation has been demonstrated to occur in numerous organisms -4). The method involved infiltration of tissue with large quantities of ptophan and subsequent bioassay for nicotinic acid with *Lactobacillus binosus* 17-5. Certain anomalous results led us to believe that other tabolites of tryptophan were interfering with the assay. Because 3-doleacetic acid (IAA) is a known plant metabolite of tryptophan (5), we ted it for possible interference with the assay, and, as described below, ind that such interference may occur under certain circumstances.

EXPERIMENTAL

The methods used were essentially those described by Snell and Wright . Stocks of *Lactobacillus arabinosus* 17-5 were carried as stab cultures in bes containing the basal medium for the nicotinic acid assay plus 1 per cent ar fortified with 1 γ of nicotinamide per tube. These were incubated for hours at 30° and were then removed to a refrigerator where they were red until use. New stab cultures were prepared every 1 to 2 weeks. Ornisms to be used in growth tests were transferred to liquid cultures conning basal medium plus 0.1 γ of nicotinamide per tube, and were incubated 30° for 18 to 24 hours immediately prior to use. 1 drop of such a culture s used as the inoculum in the growth test, since it was found that the cenugation of the culture and resuspension in saline recommended by Snell l Wright gave blanks which were not significantly smaller than ours.

Standard series were prepared containing 0, 0.05, 0.10, 0.15, 0.20, 0.30, 0, 0.50, and 1.0 γ of nicotinamide per tube. The experimental series ntained various concentrations of IAA in addition to nicotinamide. Beuse of reports of differences among various lots of IAA (6), samples of this product prepared by three different manufacturers were used. All IA solutions were adjusted to pH 7.0. Assay tubes were incubated for 72 hou at 30°; 5 ml. aliquots were then pipetted into Erlenmeyer flasks, 3 drops brom-thymol blue added, and the titration performed with 0.1 N NaOH.

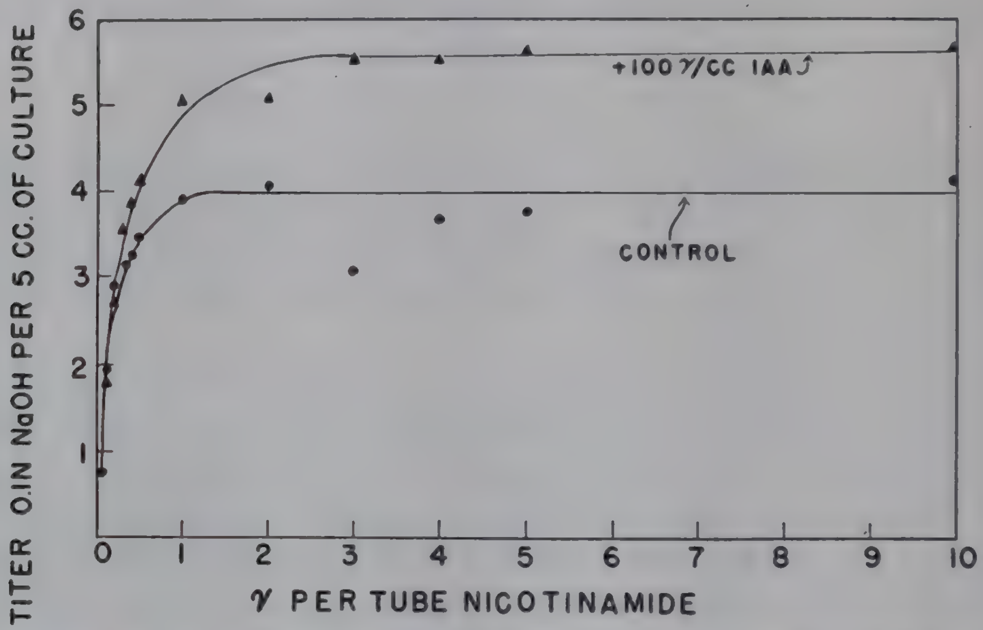

FIG. 1. The effect of 100 γ per cc. of IAA on the response of *Lactobacillus* to va ous concentrations of nicotinamide.

TABLE I

Comparative Effects of Indoleacetic, α-Naphthaleneacetic, and 2,4-Dichloropheno acetic Acids on Response of Lactobacillus to Nicotinamide

Nicotinamide per tube	Titer of 0.1 N NaOH per 5 cc. culture medium			
	Control	+ 100 γ per cc. naphthaleneacetic acid	+ 100 γ per cc. 2,4-dichlorophenoxy-acetic acid	+ 100 γ per cc indoleacetic aci
γ	cc.	cc.	cc.	cc.
0	1.06	1.24	1.10	1.17
0.10	2.25	2.28	1.13	2.13
0.30	3.81	2.81	3.48	3.51
1.0	4.20	3.86	4.35	5.35
3.0	4.64	4.57	4.96	5.65
10.0	4.35	4.18	4.58	5.20

Whereas IAA is without effect on the growth of *Lactobacillus* in the a sence of NA, it enhances the growth of the organism at the higher NA valu of the standard series. A concentration of 40 γ per cc. of IAA was bar sufficient to produce a discernible effect, whereas 100 γ per cc. yielded a co siderable growth effect. This concentration was therefore adopted as star rd in these experiments. In order to determine whether this IAA effect ould be magnified at still higher NA concentrations, new standard series ere prepared, in which the range of NA additions was carried up as high as 00 γ per tube. As may be seen from Fig. 1, the stimulatory effect of IAA maintained and even magnified at the higher levels of NA.

In higher plants, the growth-promoting effects of IAA may be duplicated y many aromatic organic acids (7). To see whether such materials could milarly substitute for IAA in the *Lactobacillus* growth effect, α-naphthaneacetic acid and 2,4-dichlorophenoxyacetic acid were applied in series parall to the control standard series and the IAA-treated series. As is seen from able I, they not only failed to stimulate significantly the growth of *Lactoacillus*, but actually depressed it at certain NA levels. The synergistic efct with NA seems, therefore, to be fairly specific for IAA.

DISCUSSION

The IAA effect described in this paper is probably of no significance in the *actobacillus* determination of NA because (*a*) the quantities of IAA present normal tissues are insufficient to cause this effect and (*b*) the range of NA oncentrations over which the IAA effect is exerted is not generally employed assays. However, if tissues are infiltrated with tryptophan in order to udy its conversion to nicotinic acid, the IAA formed enzymatically from e tryptophan may interfere with a *Lactobacillus* bioassay. In addition, nce alkaline treatment of plant proteins may release bound IAA and may so convert tryptophan to IAA (8), such treatment should be avoided in ssue to be assayed for nicotinic acid. Kodicek (9) has reported that alkane hydrolysis of grains yielded a material biologically inactive as NA which acted with the cyanogen-*p*-aminoacetophenone reagent to give an intensied color. This interference with a *chemical* determination of NA may also e attributable to IAA.

The fact that a NA-IAA interaction exists in *Lactobacillus* as well as in gher plants (10) indicates some definite metabolic connection between ese two physiologically important compounds. At present, we are unable explain the nature of the interaction.

SUMMARY

1. Indoleacetic acid, itself without effect on the growth of *Lactobacillus*, hances the growth of this organism in the presence of high concentrations nicotinamide.

2. α-Naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid, which ay replace indoleacetic acid in growth effects in higher plants, are without ch effect on *Lactobacillus*.

3. Because of the large quantities of IAA needed to produce this effect,

it is unlikely that IAA normally interferes with the microbiological assay nicotinic acid.

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ON THE METABOLISM OF $\Delta^{4,5}$-CHOLESTENONE*

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While it has been demonstrated that cholesterol is synthesized in animal issues from molecules of small size (1), notably acetic acid (2), the search for ompounds which might be intermediates in the acetate-sterol conversion as so far been unsuccessful. The possibility that cholesterol arises from ^{4,5}-cholestenone has been investigated by Anchel and Schoenheimer (3) vith inconclusive results, since the labeled cholestenone used in their experinents contained deuterium in labile positons only. On the other hand, ood evidence exists that cholestenone plays a rôle in reactions of cholesterol netabolism which lead to the formation of saturated sterols. The direct eduction of cholesterol could conceivably afford two of the epimeric dihyrosterols, namely coprosterol and dihydrocholesterol, but the change of teric configuration associated with the formation of epicoprosterol (4) reuires an intermediate which lacks asymmetry at carbon atom 3. Morever, in analogy to the chemical reduction of cholesterol, which leads to the ormation of dihydrocholesterol but not of coprosterol, it has been suggested y various investigators that the fecal sterols do not arise directly from choleserol but by way of the unsaturated ketone cholestenone as the common ntermediate. Cholestenone has not been isolated from animal tissues, but osenheim and Webster (5) succeeded in isolating the compound in considrable quantities from feces of rats and dogs. With the aid of partially abeled cholestenone Schoenheimer and collaborators have demonstrated its onversion into coprosterol in man and dog (6). These results, while strongly ndicating that cholesterol is converted to fecal sterol by way of cholestenone, ailed to reveal whether these processes occurred in the tissues or whether hey were the result of bacterial action in the intestinal tract. The belief hat coprosterol formation takes place following secretion of cholesterol into he gut has been widely held (7) in spite of the fact that this conversion has ot been unequivocally demonstrated with isolated intestinal contents.

In the present experiment the rôle of cholestenone in cholesterol metablism has been investigated further by feeding to rats labeled cholestenone hich contained deuterium uniformly distributed over the entire molecule.

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This compound was obtained as a by-product (8) of the platinum-cataly exchange reaction employed for the preparation of deuteriocholesterol (Administration of deuteriocholestenone to rats and subsequent isolation the sterols from tissues and excreta gave the results shown in Table I. In cord with the findings of Schoenheimer and collaborators (3, 6) the fecal ste following deuteriocholestenone feeding were found to contain high conc trations of deuterium, showing that in the rat also coprosterol may arise way of the unsaturated ketone. The principal result of the present inve gation is the appearance in the tissues of a sterol with a high isotope conc tration. The digitonin-precipitable sterols from liver and carcass showe significant deuterium concentration but were found on fractionation consist of two components of widely different isotope content. Choleste which comprises the bulk of the mixture, was separated by way of the bromides and was shown to contain small but significant deuterium conc trations. On the other hand, the saturated sterol, which was separated one case from the bromination mixture as the digitonide, and, in a seco experiment, after oxidation to cholestanone, had an isotope concentration least one-half that of the cholestenone fed. The identity of this satura sterol could not be established by direct isolation because of lack of suffici material but good evidence has been obtained to show that it is mainly hydrocholesterol. This is described in detail in the experimental p below. Dihydrocholesterol in small amounts is known to be present n mally in animal tissues, while coprosterol has not been isolated from a source but feces. Moreover, the finding that the fecal sterols had an isoto content less than one-third that of the saturated sterol isolated from the

TABLE I

Deuterium Concentrations in Sterols after Feeding Deuteriocholestenone Contain 5.65 Atom Per Cent Excess D

The results are expressed in atom per cent excess D.

Sterols isolated from	Experiment I		Experiment II		
	Total sterols	Cholesterol	Total sterols	Cholesterol	Sa rat ster
Blood	1.05	0.19	0.93		
Liver	1.11, 1.14*				
Carcass		0.036, 0.048	0.14	0.033	2.
Skin		0.099, 0.105			
Feces	0.72				

* After reprecipitation of the digitonide in the presence of 60 mg. of non-isoto cholestenone, the isotope concentration was 1.08 per cent D.

ıes makes it unlikely that the high isotope content of the tissue sterols was ıe to coprosterol rather than to dihydrocholesterol. The normal dihydroıolesterol content of tissues is not higher than 1 to 2 per cent (10), but in ıe present experiment, when cholestenone was fed, the content of saturated erols, particularly in the liver, must have been considerably higher. In this onnection it is of interest that Diels, after feeding cholestenone to guinea gs, noted a very low melting point for the tissue cholesterol (11). In the ght of the present findings this depression may be attributed to admixture ith larger than normal amounts of dihydrocholesterol. Thus on the basis the experiment reported here it may be suggested that the small quantities dihydrocholesterol which are ordinarily found in tissues arise from cholesrol not by direct hydrogenation but by way of cholestenone.

According to Schoenheimer (10) dihydrocholesterol cannot be absorbed om the gastrointestinal tract and its presence in the tissues therefore indites that it is formed in the internal organs. The labeled cholestenone fed the present experiment must have been readily absorbed from the intesıe. This conclusion is supported by the data which show that only 5 per nt of the administered isotope was recovered from the feces. The intesıal absorption of cholesterol is known to be a highly selective process and e ability of cholestenone to pass through the intestinal wall distinguishes is ketone from most compounds which are chemically related to cholesrol.

An alternative explanation for the present data, which has not been ruled t, is a reduction of cholestenone to cholestanone in the intestinal tract, abrption of this saturated ketone, and subsequent conversion to dihydrochosterol in the tissues. This possibility is under investigation.

The results reported here do not contain direct evidence to indicate the e of the transformation which results in coprosterol. However, since olestenone is absorbed from the intestinal tract and converted into dihydroolesterol in the tissues, it is most likely that, as Rosenheim and Starling 2) have suggested, at least the initial step in the formation of coprosterol, mely the oxidation of cholesterol to cholestenone, takes place prior to secreon into the gut.

In their experiment with partially labeled cholestenone Anchel and Schoenimer (3) found small isotope concentrations in the tissue cholesterol which d been purified by way of the dibromides. They were unable to decide ıether or not cholestenone had been reduced to cholesterol because the olestenone employed contained isotopic hydrogen in labile positions only. ıe results secured here with uniformly labeled cholestenone clearly show that e transformation of cholestenone to cholesterol occurs to a certain extent der biological conditions, but do not favor the view that cholestenone is an ermediate in the biosynthesis of cholesterol.

EXPERIMENTAL

Deuterio-$\Delta^{4,5}$-cholestenone—This compound was isolated as a by-produ of the platinum-catalyzed exchange reaction with cholesterol in D_2O-acet acid mixtures. The total ketones from the exchange reaction were isolat as *p*-carboxyphenylhydrazones and the regenerated ketones separated b chromatographic adsorption on aluminum oxide (8). The melting point the cholestenone isolated was 78–79° and was not depressed by admixture authentic cholestenone. It contained 5.65 atom per cent excess deuteriu Since this material had been treated with strong alkali during isolation, t deuterium is present in stable positions only (8).

Feeding Experiments—In the two experiments which were carried out, o rat each, weighing 150 gm., received a normal stock diet and in addition mg. of deuteriocholestenone per day for 3 days. The cholestenone was d solved in Wesson oil and mixed with the diet. The animals were killed exsanguination and the unsaponifiable material was isolated from orga and excreta by customary procedures. Total sterols were obtained by digit nin precipitation and subsequent decomposition of the digitonides by py dine (13). Bromination of this fraction yielded dibromocholesterol, whi was debrominated with sodium iodide in acetone (10).

For the separation of the saturated from the unsaturated sterols t total carcass sterols from Experiment II (0.14 gm.) were brominated ethanol according to Schoenheimer (10). Cholesterol dibromide was filter off and digitonin added to the filtrate. The digitonide which precipitat was filtered and decomposed by pyridine. The ether-soluble fraction yield after recrystallization from methanol, 3.0 mg. of a sterol, m.p. 110–11 It contained 2.75 atom per cent excess D. From the deuterium concent tions of the total sterols, cholesterol, and dihydrocholesterol it can be c culated that of the total sterols in the carcass at least 4 per cent was sat rated sterols.

Direct isolation of the saturated sterols from liver and blood was not pos ble with the small quantities available. However, good evidence for t identity of the labeled sterol with dihydrocholesterol is furnished by t following experiment. To the combined sterols (7 mg.) of liver and blo from Experiment II, which were obtained by way of the digitonides, w added 40 mg. of non-isotopic dihydrocholesterol. The mixture was oxidiz with CrO_3 to yield cholestanone (14). This was purified by adsorption alumina from petroleum ether solution and by elution with benzene (There were obtained 35 mg. of cholestanone, m.p. 128.5°, unchanged by a mixture with authentic cholestanone. The sample cont[illegible] 0.098 atom p cent excess D. In order to eliminate the possibility that contamination wi coprostanone was responsible for the isotope content, the cholestanone w mixed with an equal amount of non-isotopic coprostanone and subject again to chromatographic separation. The isotope concentration in the re-solated cholestanone was not significantly depressed (0.090 per cent D). Therefore, the sterol fraction, which was subjected to chromic acid oxidation, ould not have contained any appreciable quantities of labeled coprosterol. Moreover, since only dihydrocholesterol or epidihydrocholesterol would be xpected to yield cholestanone on oxidation with chromic acid and since pidihydrocholesterol would not have been present in the digitonin-precipitable fraction, it is reasonably certain that the saturated sterol with the high sotope concentration was indeed dihydrocholesterol.

While it has not been possible to determine directly the isotope concentra-ion of the dihydrocholesterol in liver and blood which was formed from abeled cholestenone, it can be assumed that the value lies between the iso-ope concentration of the deuteriocholestenone fed (5.65 per cent D) and that f the "dihydrocholesterol" fraction isolated from the carcass sterols (2.75 per cent D). On this basis it can be calculated that of the total sterols in iver and blood a minimum of 11 per cent was dihydrocholesterol.

The feces excreted during the feeding period in Experiment I were pooled nd yielded 12 mg. of digitonin-precipitable sterols, containing 0.72 atom per ent excess D. The material in the unsaponifiable fraction which was not precipitated by digitonin (38 mg.) contained 0.16 per cent D. The total quantity of deuterium excreted in the feces is calculated to be 0.147 mg. of D, corresponding to 5 per cent of the quantity present in the administered holestenone. The labeled cholestenone was therefore well absorbed.

SUMMARY

Cholestenone containing stably bound deuterium was fed to rats and the terols isolated from various tissues and excreta. The labeled cholestenone was well absorbed.

The highest isotope concentration was found in the saturated tissue sterols. Evidence is presented that this is due to the formation of isotopic dihydro-holesterol. The isotope concentration of the cholesterol was considerably maller but significant.

It is concluded that cholestenone can be converted to cholesterol but is ot an intermediate in the total synthesis of cholesterol. It is suggested hat the dihydrocholesterol normally found in animal tissues is formed from holesterol by way of cholestenone.

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OXIDATION OF α-HYDROXY ACIDS BY ENZYMES FROM PLANTS*

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Extensive study of the polyphenol oxidase (tyrosinase) system has been
ompted by its apparent importance as a terminal oxidase in many plants
, 2). One of the chief problems remaining unanswered for the system
what compounds serve as donors to reduce the quinone form of the sub-
rate and to maintain the shuttle between the substrate and molecular
ygen. Experimentally, ascorbic acid or glutathione can serve in this
pacity, but it has not been demonstrated that they function thus under
rmal conditions in the intact plant. Boswell (3) has suggested that
rtain amino acids may function as the natural electron donors. In their
udies, James and Cragg (4) observed that lactic, tartaric, and glycolic
ids were oxidized by the sap of etiolated barley seedlings only when as-
rbic acid was added.

Anderson (5) found that the addition of glycolic acid to cell suspensions
the colorless alga *Prototheca zopfii* stimulated an uptake of oxygen about
ice that necessary for complete oxidation of the glycolic acid added.
pparently glycolic acid acted in a catalytic capacity to enhance the oxi-
tion of endogenous metabolites. Kolesnikov (6) reported the isolation
glyoxylic acid from freshly ground barley leaves. He found (7) that
e addition of glycolic acid to ground barley leaves caused the uptake of
to 15 times the amount of oxygen necessary for its oxidation to glyox-
ic acid. This was accompanied by the disappearance of chlorophyll from
e suspensions.

In our survey of possible electron donors in the polyphenol oxidase sys-
m the only compounds observed to stimulate oxygen uptake by centri-

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47, and was presented by C. O. Clagett and by N. E. Tolbert and R. H. Burris at
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cember 30, 1947.

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fuged sap from soy bean leaves were catechol, dihydroxy-L-phenylalani lactic acid, and glycolic acid. Our attention was drawn to lactic acid a substrate and more particularly to glycolic acid, because it supported v active respiration and had been studied little.

EXPERIMENTAL

Methods and Materials—Measurements of oxygen consumption w made in Warburg respirometers at 30°. 1 ml. of 0.1 M phosphate buf 1 ml. of centrifuged sap or diluted enzyme preparation, and 1 ml. of 0 M substrate were added to each flask; 0.15 ml. of 20 per cent potassium droxide was placed in the central alkali well.

The enzyme was prepared by grinding young succulent leaves wit Nixtamal mill in the cold and expressing the sap through cheese-clo The sap was adjusted to pH 8.0 with N sodium hydroxide and cen fuged (about 20,000 times gravity for 3 minutes) in a small Beams a driven centrifuge (8); a clear supernatant liquid was obtained.

Most of the substrates used were commercial preparations. α-Hydro n-butyric acid was prepared from the α-bromo derivative by the meth of Naumann (9). The *l*-lactic acid was supplied by M. J. Johnson of t department.

Results

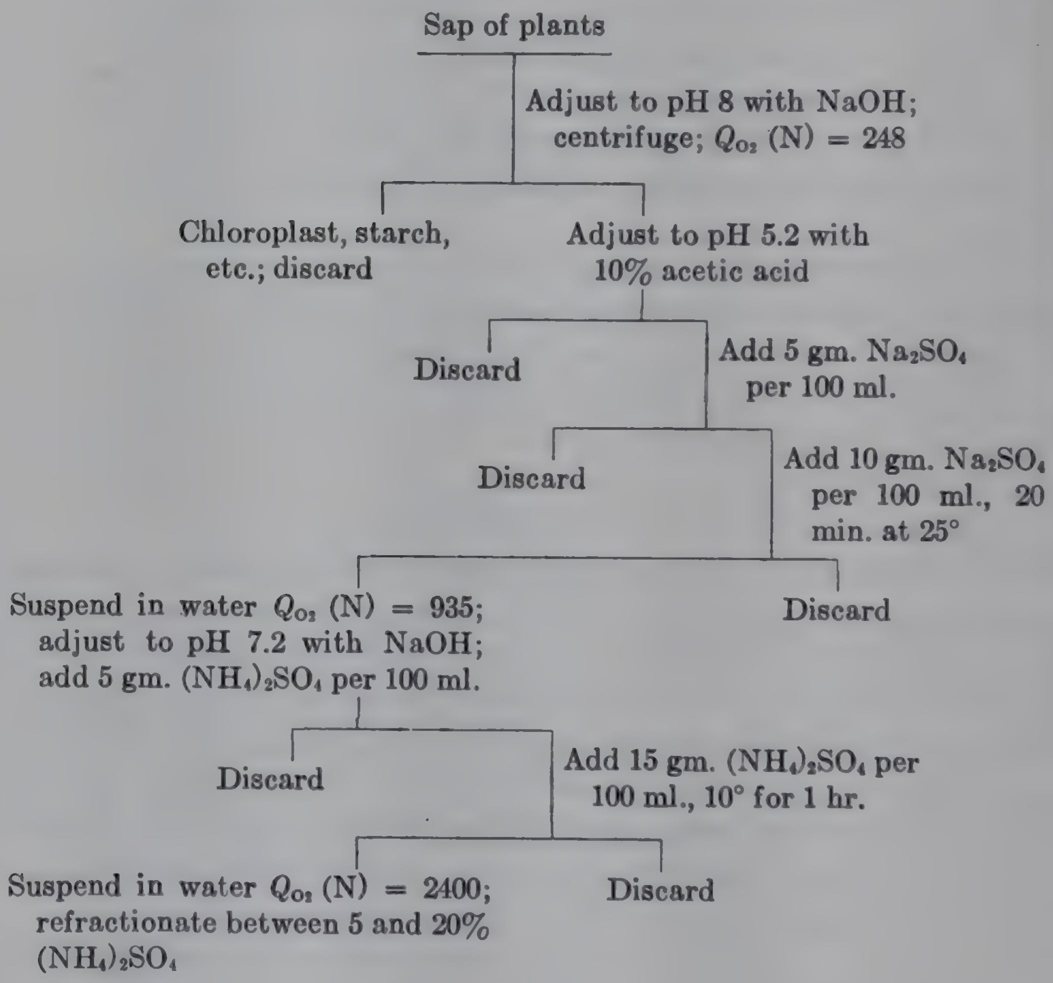
Distribution of Enzyme—The α-hydroxy acid oxidase of plants is wid distributed in nature. Stutz, MacVicar, and Stauffer (unpublished da surveyed representatives from a large number of plant orders for th ability to oxidize *dl*-lactic acid and found activity in preparations from leaves of the following plants: barley, elegans lily, sunflower, squa fuchsia, soy bean, cabbage, snapdragon, balsam, mint, tobacco, Sw chard, hollyhock, Japanese yew, club moss, moss, honeysuckle, white o and grape. The following plants showed no activity: vanilla, German i philodendron, celery, sedum, geranium, peony, marchantia, and spi gyra.

Effect of Light on Formation of Enzyme—Preliminary experiments dicated that the enzyme is present in an active form only after exposure the plants to light. No enzyme could be demonstrated in the sap fr wheat embryo, barley embryo, 24 or 96 hour-germinated barley, or g minated clover. Etiolated barley or beans (5 to 10 days after plantin did not contain the enzyme system. However, it was present and act after the etiolated beans were exposed to 15 hours and the etiolated bar to 4 hours of winter light in the greenhouse. Exposure of the sap fr etiolated barley or beans to light did not activate the enzyme. Within range tested, the age of green barley plants did not markedly affect th ility to oxidize glycolic acid. However, the older the plant material, e more stable was the enzyme system in the expressed sap. Green to-cco plants which had been kept in the dark for 9 days, until the leaves d yellowed and had started to decompose, yielded very active enzyme eparations.

In our tests the centrifuged sap of soy bean, tomato, and potato leaves d a Q_{O_2} (N) of about 30 in the absence of added substrates. Centri-ged sap of barley and tobacco leaves prepared in the same manner owed no respiration in the absence of added substrates; this fact prompted e further use of these two plants. Tobacco was usually the plant of oice, for the enzyme preparations from it were more stable than those om barley. Washed chloroplasts from tobacco and barley had Q_{O_2} (N) lues of 40 to 50 and 60 to 70 respectively in the absence of added sub-ates, and showed very little response to the addition of glycolic or lac- acid.

Concentration of Enzyme—Blanchard *et al.* (10) used a combination of dium sulfate, heat treatment, and ammonium sulfate precipitation in the rification of L-amino acid oxidase from animal tissues. A similar scheme s used to obtain a concentrate of our α-hydroxy acid oxidase. Tobacco f sap was extracted by grinding in a Nixtamal mill and pressing through eese-cloth. The sap was cooled immediately to 0° and kept cold, ex-pt during centrifugation. It was adjusted to pH 8.0 and centrifuged to nove chloroplasts, cell fragments, and starch granules. The clarified was adjusted to pH 5.2 with 10 per cent acetic acid (acetic acid is not ed as a substrate), and the precipitated material was removed by cen-fugation. 5 gm. of sodium sulfate per 100 ml. of sap were added, and a all amount of colorless precipitate containing little activity was removed. gm. of sodium sulfate per 100 ml. of original sap were then added to the pernatant and the solution was held for 20 to 30 minutes at room tem-rature before centrifugation. Most of the original activity was con-trated in this precipitate. The precipitate was suspended in a volume water equal to one-half the original volume of sap; the pH was adjusted 7.2 with sodium hydroxide solution. 5 gm. of ammonium sulfate per ml. of solution yielded a precipitate of low activity which was dis-ded. 15 gm. of ammonium sulfate were added per 100 ml. of the super-ant; the solution was refrigerated for 1 hour at 10°, then centrifuged, l the supernatant discarded. The precipitate was suspended in a vol-e of distilled water equivalent to a fourth the volume of the original . The residual ammonium sulfate in the solution appeared to prevent ilage by microorganisms. The preparations retained most of their ivity for several days at 5°. The addition of substrate had no apparent bilizing effect on the enzyme. Heat treatment was ineffective for purification. Attempts to purify the enzyme by adsorption and elution fr Hyflo Super-Cel were unsuccessful. Bach, Dixon, and Zerfas (11) us freshly precipitated calcium phosphate gel as an adsorbent in concentr ing yeast lactic dehydrogenase and followed this by elution of the enzy with alkaline buffer containing ammonium ions. Calcium phosphate adsorbed about 66 per cent of the enzymatic activity of the α-hydroxy a oxidase at pH 8.0. Proof that the adsorbed enzyme retained activity w obtained by suspending a portion of the gel in buffer and determining t rate of oxidation of sodium glycolate. No elution was achieved with pho phate buffers between pH 5.0 and 8.0, and attempts to elute the enzy with phosphate buffers containing ammonium ions or traces to 0.5 p cent pyridine inactivated the enzyme.

Scheme for Purification of α-Hydroxy Acid Oxidase


Sap of plants
Adjust to pH 8 with NaOH; centrifuge; Q_{O_2} (N) = 248
Chloroplast, starch, etc.; discard
Adjust to pH 5.2 with 10% acetic acid
Discard
Add 5 gm. Na_2SO_4 per 100 ml.
Discard
Add 10 gm. Na_2SO_4 per 100 ml., 20 min. at 25°
Suspend in water Q_{O_2} (N) = 935; adjust to pH 7.2 with NaOH; add 5 gm. $(NH_4)_2SO_4$ per 100 ml.
Discard
Discard
Add 15 gm. $(NH_4)_2SO_4$ per 100 ml., 10° for 1 hr.
Suspend in water Q_{O_2} (N) = 2400; refractionate between 5 and 20% $(NH_4)_2SO_4$
Discard

The method outlined for concentrating the enzyme was used succe fully with tobacco leaves from mature plants and with soy bean leav No success was attained when very young tobacco or barley plants we used; the activity was invariably destroyed when the pH was adjusted

2. No explanation is apparent for the instability of the enzyme in very ung plants.

Optimum pH—The exploratory experiments were conducted at pH 5.9, this is within the pH range of the expressed sap. Preliminary experi- ents on glycolic acid oxidation indicated the need of a wider range of uffering than is effectively supplied by phosphates. A composite buffer lution containing potassium acid phthalate, phosphoric acid, and sodium rate, 0.1 M with respect to each component, was used. An electrometric ration curve of the buffer solution indicated almost linear buffering pacity over the desired range of pH 4 to 11. It was soon found that the H optimum for glycolic acid oxidation was above the buffering range of thalate; so the phthalate was omitted from the buffer used in the deter- ination of the pH optima reported here. The borate component of this iffer had a slightly inhibitory effect (10 per cent inhibition as compared th phosphate alone) on the rate of oxidation but did not alter the total ygen consumption per unit of glycolic acid.

Fig. 1 illustrates the effect of pH on the rates of glycolic acid and lactic id oxidation catalyzed by enzyme solutions concentrated by salt pre- pitation. A similar curve was obtained for the rates of oxidation of ycolic acid when the crude sap was used, except that the range on either le of the optimum was increased. Activity on glycolate is approxi- ately constant between pH 7.8 and 8.6. The optimum oxidation of ctate was observed at pH 7.6. As there is an appreciable drop in pH ring the oxidation of glycolate, the values plotted are the average of the itial and final pH in each flask. In the lactate oxidation there is no pH ange. The rates of oxygen uptake for the period 5 to 15 minutes after ding the enzyme are plotted. Below pH 6.0 and above 9.3 there is no easurable exchange with glycolate during the first 5 minutes after adding e enzyme; after this, however, considerable activity is observed beyond ese limits of pH.

Effect of Enzyme Dilution—The effect of dilution of the enzyme was easured as an indication of the participation of cofactors in the oxida- on of glycolate. If such factors exist, dilution with buffer to a point here the cofactors become limiting should result in a decrease in activity eater than that proportional to the enzyme dilution. Dilution did not crease the activity per unit of enzyme (Fig. 2). At enzyme concentra- ons equivalent to more than 25 per cent centrifuged tobacco sap the activ- was so great that diffusion of oxygen became the limiting factor. In cceeding experiments the enzyme was always diluted in order to insure easurements on a linear portion of the dilution curve.

Effect of Temperature—Within limits, increase in temperature increases e velocity of reactions catalyzed by enzymes. The effect of tempera-

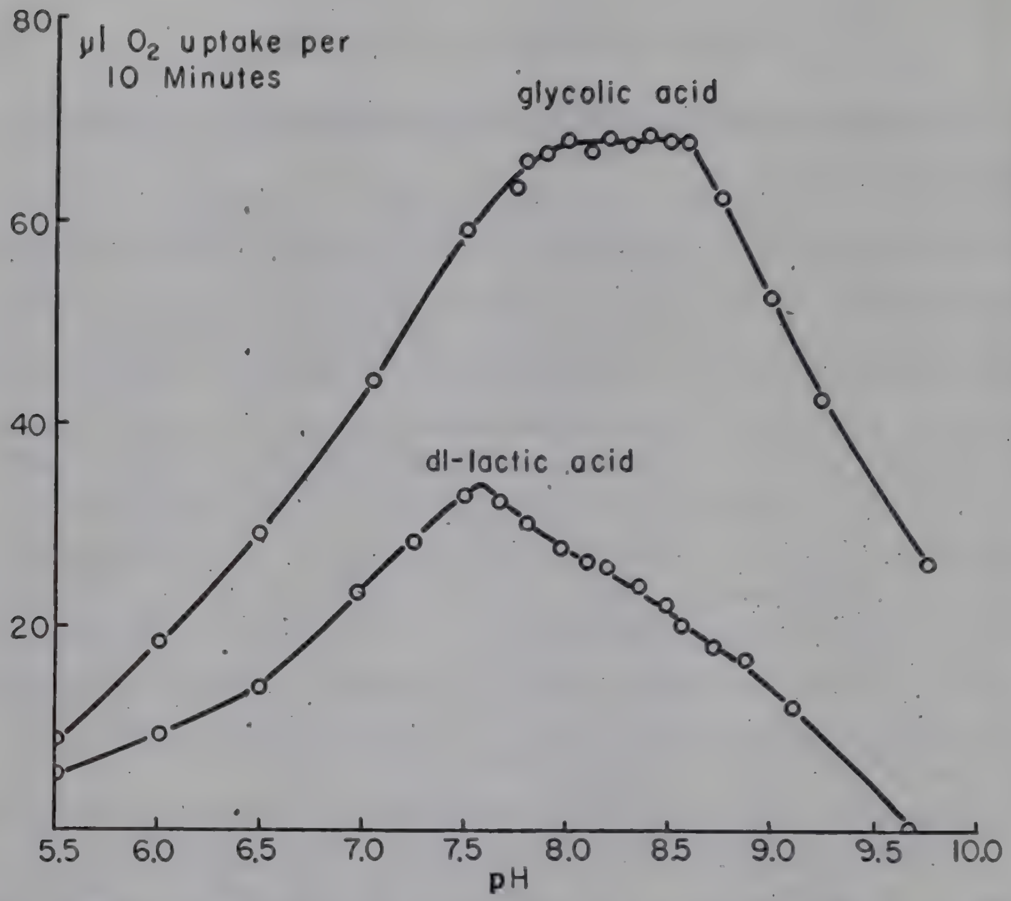


FIG. 1. Effect of pH on the rate of oxidation of glycolic acid and lactic acid the concentrated enzyme from tobacco sap. The flasks contain 0.5 ml. of 0.02 neutralized glycolic acid or 0.08 M neutralized *dl*-lactic acid, 1.0 ml. of phosphat borate buffer, 1.0 ml. of water, and 0.5 ml. of diluted enzyme preparation.

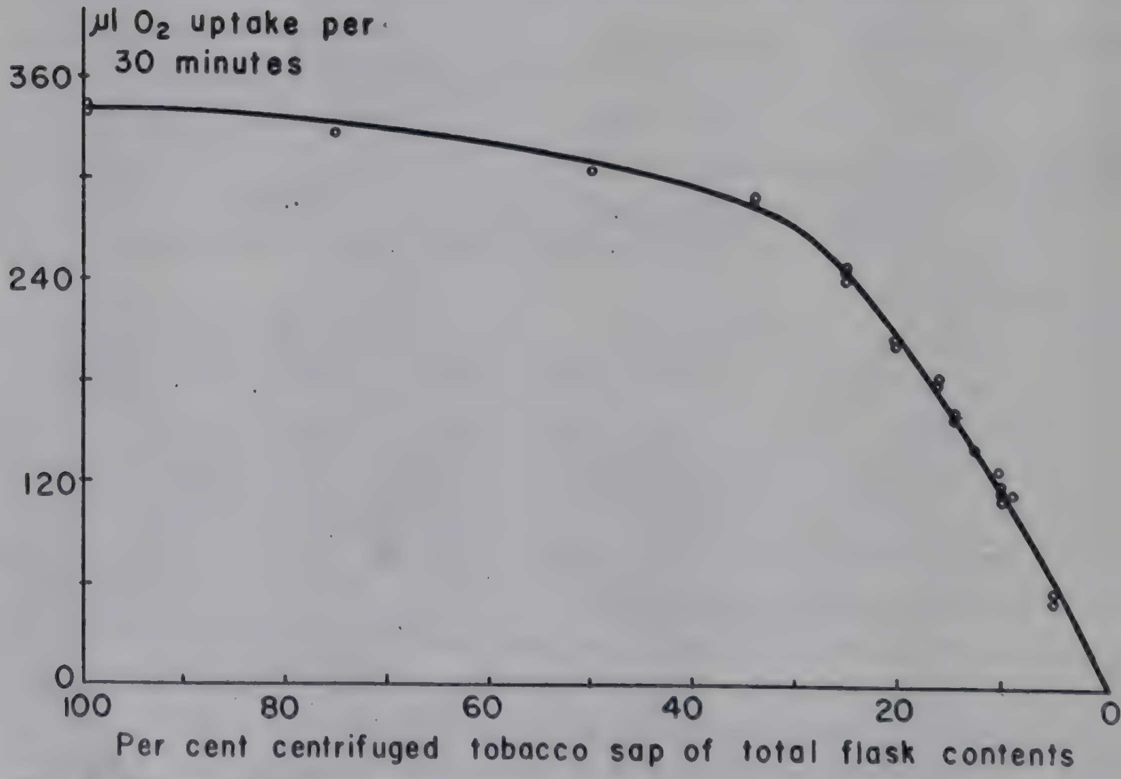


FIG. 2. Effect of dilution on activity of the α-hydroxy acid oxidase from tobac sap.

re on the rate of oxidation of glycolic acid by barley sap and by the con- ntrated enzyme from tobacco sap was determined. Table I illustrates e results of such experiments.

The temperature coefficients (increase in activity per 10° temperature e) of the crude sap and the partially purified preparation were approx- ately 1.17 between 20–35° within the limits of experimental error (Table Measurement of the initial rate of oxidation shows the maximum ac- ity of the enzyme as it exists in barley sap to be between 35–40°; above is it is rapidly destroyed. The concentrated enzyme preparations from oacco sap were rapidly inactivated above 35°. The molar energy of tivation calculated from these data with the Arrhenius equation is 2.3 ±

TABLE I

Temperature Coefficients for Oxidation of Glycolic Acid

Temperature interval	Temperature coefficient	
	Barley sap	Salt ppt. enzyme, tobacco
°C.		
10–20	1.33	
20–30	1.12	
30–40	1.17	
40–50	0.14*	
25–35		1.19
35–45		0.22*

Enzyme destroyed by heat at the higher temperatures.

kilocalories. This value is among the lowest reported for enzymes (12) l is in the range of that reported for tyrosinase.

ubstrate Concentration—The effect of substrate concentration was de- mined and the data analyzed according to the method of Lineweaver l Burk (13). Oxidation rates of barley and tobacco leaf sap were asured at concentrations of sodium glycolate between 4.2×10^{-4} and 3×10^{-2} M. Below the 4.2×10^{-4} M concentration, oxidation rates were and erratic. The Michaelis constant for the oxidation of sodium colate was found to be 2.4×10^{-3} M.

xygen Consumption per Unit of Substrate—The oxygen consumption entrifuged barley and tobacco leaf saps was negligible in the absence ubstrate. In the presence of glycolic acid the equivalent of 1 mole of gen per mole of substrate was used, and the oxidation of *l*-lactic acid uired 0.5 mole of oxygen per mole of substrate (Fig. 3).

he oxygen consumption with glycolate was equivalent to the oxidation lycolic acid to oxalic acid. As oxalic acid is found in quantity in many

plants, it seemed a likely product. Measurement of respiratory
tients indicated a release of varying amounts of carbon dioxide, depen
to a large extent upon the strength of the acid used to release the bo
CO_2. The evolution of CO_2 is incompatible with the idea that oxalic
is the exclusive end-product of the oxidation of glycolic acid. The m
anism of the oxidation will be described in a further publication.

It is realized that the enzyme concentrates used in these studies
not homogeneous, and any statements on specificity are open to some q

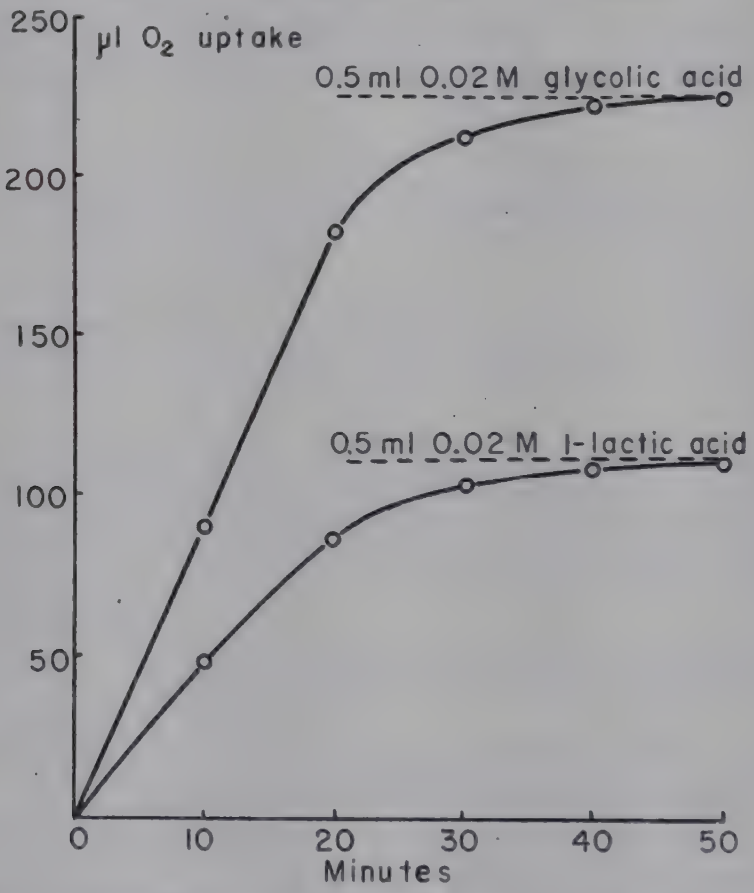


FIG. 3. Oxygen uptake for the oxidation of glycolic acid and *l*-lactic acid as c
lyzed by the concentrated enzyme from tobacco.

tion. The tobacco sap and the concentrated enzyme contained a catal
a polyphenol oxidase capable of oxidizing catechol and dihydroxy-L-phe
alanine, and an enzyme system catalyzing the oxidation of *o*-phenyl
diamine. Under the conditions of our experiments the oxidation of
colic acid appears to be independent of these other enzymes, for its ox
tion occurs at about the same rate in the presence or absence of substr
for the other enzymes. The enzyme preparations decomposed hydr
peroxide rapidly and the addition of catalase from animal tissue did
change the rate of oxidation of glycolic acid. The presence of cyanid
azide, which poisons catalase, does not alter the ratio of 1 mole of oxy
uptake per mole of glycolic acid oxidized. The addition of ethyl alc

es not change the rate of oxygen uptake or the total oxygen uptake ap- eciably; if hydrogen peroxide were formed in the presence of catalase d ethyl alcohol, the oxygen uptake should be doubled (14). Appar- tly peroxidase activity is not involved in the oxidation. The concen- ated enzyme preparation does not oxidize ascorbic acid, whereas the sh sap does; ascorbic acid inhibits the oxidation of glycolic acid, par- ularly at a pH below 8.0.

Substrate Specificity—Concentrated enzyme preparations from tobacco ves did not oxidize *l*-malic, *d*- or *l*- or mesotartaric, DL-glyceric, or α-hy- oxyisobutyric acid. The preparations were specific for the L isomer of tic acid. The oxidation of DL-α-hydroxy-*n*-butyric acid was very slow. gher homologues of the straight chain α-hydroxy acids were not tested.

TABLE II

Comparison of Oxidation of l-Lactic Acid and Glycolic Acid by Various Enzyme Fractions

Preparation		Rate of oxidation, μl. O_2 per 30 min.		$\frac{\text{(Lactic)}}{\text{(Glycolic)}}$
		Glycolic acid	Lactic acid	
A	Sap	192	119	62
	Na^+ ppt.	119	77	64
	NH_4^+ “	218	164	75
B	NH_4^+ “	203	146	72
C	Sap	157	121	77
	Na^+ ppt.	71	63	88
	NH_4^+ “	176	149	85

A comparison of the rate of oxidation of *l*-lactic acid and glycolic acid whole sap and by sodium sulfate- and ammonium sulfate-precipitated zyme is shown in Table II. The ratio of rates of oxidation of lactic and colic acids has not been changed by partially inactivating the enzyme eparation by heat or by lowering the pH to 4.5 to 5.0 for short periods. separate enzymes are responsible for the oxidation of these two acids, ey must have similar solubility and stability characteristics. When th lactic and glycolic acid substrates were added together, the initial o order reaction rate was only slightly greater than that obtained from oxidation of glycolic acid alone, and the total oxygen uptake was the n of the glycolic and lactic acid oxidations alone. If there were two zymes, each acting on one of the substrates, the initial rate of oxidation ould have been about the sum of that obtained when the enzyme prep- tion was used with each substrate separately. Further purification of enzyme is necessary before the question of enzyme specificity can be ablished finally; however, the data at hand suggest that one enzyme is ponsible for the oxidation of these two α-hydroxy acids.

Inhibitors—Sodium azide had little influence on the rate of oxidation glycolic acid; even at a concentration of 0.01 M, the inhibition was than 10 per cent. Cyanide at 0.1, 0.01, and 0.001 M concentrations st ulated oxidation somewhat with 0.0067 M glycolate and inhibited 13 25 per cent with 0.0017 M glycolate. At a final concentration of 0.01 sodium malonate gave 30 per cent, sodium iodoacetate 48 per cent, hydroxylamine 30 per cent inhibition of glycolate oxidation. These hibitors were virtually without effect at a concentration of 0.001 M.

DISCUSSION

The literature on plant respiration has been covered in the recent views by Stiles (15) and James (16). Both reviewers have cited instan in which the polyphenol oxidase system has been assigned a function a terminal oxidase. Our studies were undertaken with the object of taining more information on the chain of reactions which must prec the action of the terminal oxidase in the respiration of carbohydrates plants. Although we observed an enzyme system capable of oxidiz α-hydroxy acids at a rapid rate, it was not possible to demonstrate tha required either quinones from polyphenols or dehydroascorbic acid as el tron acceptors. Apparently the transport of electrons to molecular o gen proceeded independently of the polyphenol or ascorbic acid oxidase

James and Cragg (4) demonstrated an increased oxygen uptake by of etiolated barley seedlings in the presence of ascorbic acid and glyco lactic, or tartaric acid. We have confirmed these observations with et lated seedlings, but in contrast the oxidation of glycolic and lactic ac by preparations from green leaves has always been independent of presence of ascorbic acid. The α-hydroxy acid oxidase reported in t paper was never found in etiolated plants. The concentrated enzyme fr green tissue was inactive toward ascorbic acid; its oxidation of glyc acid was somewhat inhibited by ascorbic acid.

The character of the enzyme has not been determined. The prepa tions used in these studies have been relatively crude. The absence o heavy metal component was indicated by the lack of azide and cyan inhibition. Sodium malonate, sodium iodoacetate, and hydroxylam all inhibited the reaction to some extent. The enzyme is stable to dia sis at 5° for long periods; this indicates the absence of any easily remova cofactors. The enzyme plus glycolate and diphosphopyridine nucl tide does not decolorize methylene blue anaerobically. The enzyme similar to the L-amino acid oxidase of rat kidney isolated by Blanch *et al.* (10) in substrate specificity and pH of optimum activity. It fers from the animal enzyme in heat stability, solubility, and effect of length of the carbon chain of the α-hydroxy acid on the rate of respirati lso, the plant extracts were incapable of mediating the oxidation of L- nino acids.

The rôle of the enzyme system in the economy of the plant is not ap- arent. The fact that the active enzyme system is found only in the hlorophyllaceous plant may be suggestive of a possible function in the hotosynthetic reactions.

SUMMARY

1. The occurrence of an enzyme system in plants capable of oxidizing -hydroxy acids was demonstrated.

2. Preliminary studies indicated its presence in green leaves of a large umber of plant orders but not in etiolated seedlings or in embryos.

3. 1 mole of oxygen is used in the oxidation of 1 mole of glycolic acid nd 0.5 mole in the oxidation of *l*-lactic acid. Apparently hydrogen per- xide is not formed in the reaction.

4. The pH optimum for lactic acid oxidation is 7.6. With glycolate ne activity is maximum and nearly constant between pH 7.8 and 8.6; ne temperature coefficient is 1.17 and the energy of activation is 2.3 ± 4 kilocalories; the temperature optimum is 35–40°, and the *Michaelis nstant* is approximately 2.4×10^{-3} M. No easily dissociable cofactors ppear to function in the oxidation.

5. A 10-fold concentration of the enzyme was achieved from tobacco af sap by a combination of isoelectric precipitation, sodium sulfate, and nmonium sulfate precipitation.

6. The enzyme appears to be specific for *l*-α-hydroxymonocarboxylic cids.

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THE EFFECT OF PYRIDOXINE DEFICIENCY ON THE ABSORPTION OF IRON BY THE RAT

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The factors which govern the absorption of iron are not well understood. ıe current theory of iron absorption assigns to bodily need for iron the ief rôle in the regulation of iron absorption (1, 2). Conclusive evidence ıeeded, however, to demonstrate the correctness of such a view.

In an earlier study from this laboratory (3), it was demonstrated that the gh serum iron values and hemosiderosis of the liver and spleen, which e characteristic of pyridoxine deficiency in swine, do not appear when e experimental animals are fed a diet very low in iron content, even ough all other manifestations of pyridoxine deficiency develop. This servation indicates that, at least in pyridoxine-deficient swine, iron con- ues to be absorbed even though it cannot be utilized and in spite of the t that the body stores are replete with iron.

Although it is evident that iron absorption in pyridoxine deficiency may present unusual circumstances which are of no significance in the normal imal or in other types of deficiency, it was thought important to study e problem further. One point needing elucidation is whether or not iron sorption is normal, reduced, or even possibly increased in the face of ridoxine deficiency as compared with normal. Since the answer to this estion could only be obtained by measuring the total iron content of perimental animals, and since this could not be done easily in swine, the was chosen for the experimental studies to be described. The rat differs m swine and dogs in that little or no anemia develops when pyridoxine ficiency is produced, even though various other signs of this deficiency do 5).

Materials and Methods

Experiment I—Weanling Sprague-Dawley rats were used for this study. om the outset they were divided into three groups: Group A, six rats; oup B, ten rats; and Group C, ten rats. All rats were placed on a basal t consisting of crude casein (Sheffield "new process") 27 per cent, sugar

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* Aided by grants from the United States Public Health Service and The Upjohn npany, Kalamazoo, Michigan.

58.0 per cent, Snowdrift, a hydrogenated vegetable oil, 5.0 per cent,
5.0 per cent, salt mixture[1] 4.0 per cent, and cod liver oil 1.0 per cent.
min E in the form of mixed tocopherols[2] was incorporated into the
liver oil and mixed with the diet in the amount of 1.5 mg. per kilo of

Group A (controls fed *ad libitum*) and Group C (pyridoxine-defic
rats fed *ad libitum*) received the following vitamin supplements (mg.
kilo of diet): thiamine 10, riboflavin 10, calcium pantothenate 40, ch
chloride 500, nicotinic acid 100, *p*-aminobenzoic acid 600, and ino
1000. These were mixed with the diet. In addition, Group A rece
50 γ per rat per day of pyridoxine hydrochloride dissolved in the
citrate solution.

Group B (restricted controls) received their vitamin B supplemen
the form of powdered yeast, making up 4 per cent of the diet. Vita
A, D, and E were given as in the other groups.

All three groups received the equivalent of 1 mg. of iron per day in
form of 11.0 mg. of ferric citrate in aqueous solution administered o
by pipette every other day. Iron administration was not started unti
first signs of pyridoxine deficiency appeared, *i.e.* untidy fur, red whisk
and xanthurenic acid excretion in the urine (12). This was done in o
to rule out any absorption during the period before the pyridoxine
ficiency began exerting its effect.

Group B was included in order to obviate any results due to differe
in weight of Groups A and C.

Hemoglobin determinations were made about every 2 weeks with b
obtained by clipping the tail. The hemoglobin was determined as
hemoglobin by means of the Evelyn photoelectric colorimeter.

The rats were kept on this régime for 12 weeks, at which time they
all sacrificed by ether anesthesia and stored in the refrigerator until as
Iron feeding was discontinued 4 days before the rats were to be sacrifi
in order to permit elimination of any unabsorbed iron from the gut.

Experiment II—A second experiment was carried out in which thir
Sprague-Dawley rats were divided into two groups: Group A, cont
six rats; Group B, pyridoxine-deficient, seven rats. The dietary in
of rats of Group A was restricted in order to keep their weights appr
mately the same as those of Group B. Group II-B rats were abou
week older than those in Group I-C when they were started on the exp
mental diet. The care, feeding, etc., was exactly the same as in Exp
ment I, except that Experiment II was carried on for 8 weeks instea

[1] The salt mixture had the following percentage composition: $NaHCO_3$
$CaHPO_4$ 25.0, $MgSO_4$ 14.0, K_2CO_3 4.65, KCl 31.2, $CuSO_4$ 0.24, $MnSO_4$ 0.15, KI
NaF 0.03.

[2] Parke, Davis and Company.

All rats in Group I-C manifested symptoms of severe pyridoxine deency, while those in Group II-B never exhibited extreme outward sympns of the deficiency, although they showed a considerable excretion of thurenic acid.

The determination of the total iron in such biological material as a whole presents considerable difficulties due to the large amount of material l to the large quantities of salts present in the animal carcass, notably osphates and calcium. Since wet ashing is entirely impractical for such rge animal, dry ashing was the only recourse open. Hence the carcasses re dry ashed in large porcelain trays, the heating being started at 100–)° until danger of excessive foaming was passed; then the temperature s raised to 600° for 12 to 18 hours. The ashing proceeded smoothly, l the ash was easily dissolved in 25 ml. of concentrated HCl with gentle rming.

It has been suggested that dry ashing leads to loss of iron through volazation as $FeCl_3$ (6). Experience in this laboratory,[3] as well as that of bian *et al.* (7), would indicate that there is very little, if any, loss by this ans. It is quite likely that the losses suggested were due to lack of full or development as a result of phosphate interference.

The dissolved ash was transferred to a 100 ml. volumetric flask and made volume with double distilled water. 2 to 5 ml. aliquots of this were nsferred to 50 ml. volumetric flasks and made to volume with double tilled water. Aliquots (1 to 2 ml.) were then taken for the colorimetric ermination of iron, with use of the Evelyn photoelectric colorimeter l α,α-dipyridyl as the color reagent. The method finally used was a nbination of the methods of Kitzes, *et al.* (8) and Woiwod (9) modified suit the ashed material.

The high concentration of salts, expecially calcium and phosphates, in ash from a whole rat interferes in the determination of iron by the ial colorimetric methods. A number of procedures have been suggested overcome this interference, such as by boiling the ash with NaOH (10), h HCl or HNO_3 (11, 12), by precipitation of the iron as the sulfide owed by filtration and resolution in acid (6, 9), or by fusing the ash with cium or sodium carbonate (13). These are all designed to hydrolyze pyrophosphates, presumably formed during ashing, which produce nonized complexes with iron and thus retard or prevent its combination h the color reagent. However, these methods all involve considerable litional manipulation, which increases the chances for loss or contamiion with iron and also the addition of reagents which are high in iron, ably NaOH. In view of the disadvantages cited, these methods are erally quite unsatisfactory. α,α-Dipyridyl has been found more satis-

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Unpublished.

factory for use with this type of material. It forms a more stable comp than thiocyanate and is less sensitive to interference from phosphates th is either thiocyanate or *o*-phenanthroline. As a result the color devel more rapidly and more completely. If 24 to 48 hours are allowed for co development with α,α-dipyridyl, reproducible results are obtained wh compare well with those obtained with the titrimetric dichromate meth of Bernhardt and Skeggs (14) as modified by Sill[4] to prevent air oxidati

The method employed in this work was as follows: Suitable aliquots the diluted rat digest solution were pipetted into iron-free test-tubes. this was added 1.0 ml. of freshly prepared (10 per cent) sodium sul solution followed by thorough mixing. Then 0.5 ml. of a 1.0 per cent so tion of α,α-dipyridyl in 0.1 N HCl was added and mixed well. A sufficie volume of saturated sodium acetate was then added to make the pH ba (red) to Congo red paper. Usually 2 ml. were required. Double d tilled water was then added volumetrically to make the total volume 10 ml. The tubes were shaken, covered, and set aside for at least 24 ho before reading. The blank was prepared in the same manner as the sa ples, except that water was used in place of the aliquot of sample.

The readings were made with use of the Evelyn photoelectric colori eter and Filter 520 after adjusting the blank to 100.

In the second experiment total body copper determinations were a made on the rat digest solutions by a modification of McFarlane's (1 method as suggested by Tompsett (16) for urine, feces, and other materi high in iron and calcium. 0.5 ml. of the concentrated rat digest soluti was pipetted into acid-washed test-tubes. To this, 4.5 ml. of saturat sodium citrate solution were added to bind the calcium in a soluble co plex. To this were added, in the following order, 1 ml. of saturat $Na_4P_2O_7$ (sodium pyrophosphate) solution, 2 ml. of concentrated NH_4O and 1.0 ml. of 0.1 per cent sodium diethyl dithiocarbamate solution, w mixing after each addition. Water was then added to give a total volu of 15 ml. The color was read immediately with the Evelyn photoelect colorimeter with Filter 440.

$$\frac{\text{Concentration}}{D} = K$$

$$\text{Concentration (mg. per rat)} = D \times K \times \frac{100}{\text{ml. sample}}$$

Results

The data for both experiments are presented in Table I. The hem globin, total body iron, and iron per 100 gm. of body weight are express s the mean for the group along with the respective standard deviations. he data were analyzed statistically and the significance of the difference etween the means was tested by Fisher's t test. The increase in iron per 00 gm. of body weight in the pyridoxine-deficient Group I-C is significant hen compared with either Group I-A (*ad libitum* controls, t = 3.94) or roup I-B (restricted controls, t = 4.31). The same is true in comparing he pyridoxine-deficient Group II-B with Group II-A (restricted controls, = 4.48). The differences in total body iron between Groups I-A and -C and Groups II-A and II-B are not significant, but this is due to the rger body weight of the controls (*i.e.*, 325 gm. and 226 gm., respectively r Groups I-A and II-A as compared to 213 gm. and 192 gm., respectively r Groups I-C and II-B. The increase in total iron in Group I-C as compared to Group I-B, however, is significant at the 1 per cent level (t = 62).

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[4] Sill, C., unpublished personal communication.

TABLE I

Iron and Copper Content of Rats in Various Experimental Groups

Group No.	Condition	No. of rats	Mean weight	Hb	Total body iron	Iron per 100 gm.	Total body copper	Copper per 100 gm.
			gm.	*gm. per cent*	*mg.*	*mg.*	*mg.*	*mg.*
I-A	*Ad libitum* controls	6	325 ±3.8	14.47 ±0.49	16.0 ±1.1	4.93 ±0.37		
I-B	Restricted controls	10	236 ±6.8	13.51 ±0.58	12.1 ±1.1	5.12 ±0.52		
I-C	Pyridoxine-deficient	10	213 ±21.8	13.92 ±1.41	17.5 ±4.2	8.20 ±2.20		
I-A	Restricted controls	6	226 ±10.0	16.95 ±0.55	11.03 ±0.8	4.97 ±0.35	1.30 ±0.07	0.58 ±0.05
-B	Pyridoxine-deficient	7	192 ±21.6	15.85 ±1.08	11.85 ±1.3	6.49 ±0.75	1.98 ±0.36	1.03 ±0.17

One rat in Group I-C was omitted from the statistical analysis because oth its total iron (51.5 mg.) and iron per 100 gm. (26.1 mg.) were found be 8 standard deviations from the mean. Since this rat exhibited more vere symptoms of deficiency than the others, it seemed justifiable to conder the animal separately.

The pyridoxine-deficient Group II-B also shows a significant increase in oth total copper and copper per 100 gm. when compared to control Group -A (t = 4.47 and 6.25, respectively).

The hemoglobin values show no significant reduction in the pyridoxine-ficient groups as compared with the controls.

DISCUSSION

The results of these experiments indicate that the absorption of iron
increased during pyridoxine deficiency. This is an interesting finding
view of the fact that Granick (2) and Whipple and coworkers (1, 17) ha
suggested that the intestinal mucosa has the property of accepting or
jecting iron, depending on the state of the iron stores in the body. A
cording to this hypothesis, since in pyridoxine deficiency the synthesis
hemoglobin is retarded and the serum iron is elevated, it would be expect
that the need of the body for iron would be reduced. Yet, as has be
demonstrated in these experiments, iron continues to be absorbed even
the extent that the total body iron is increased. This condition wou
seem to present an exception to the "mucosal block" theory. Duba
Callender, and Moore (18), with use of the isotope technique for studyi
iron absorption, found a similar situation to exist in patients with untreat
pernicious anemia, refractory anemia, and hemolytic anemia, even thou
the tissues were replete with iron in these conditions. These authors su
gest that the mucosal block theory must be thought of only in relati
terms.

Many factors which play a rôle in the regulation of iron absorption
certainly still unknown. It is possible that the theory of Granick, Whipp
and their associates describes the mechanism operating under normal co
ditions but that this mechanism breaks down and does not function in su
conditions as pernicious anemia and pyridoxine deficiency. For examp
apoferritin, a protein, has been postulated to play a central rôle in iron
sorption (2). Pyridoxine has been shown to be involved in protein m
tabolism as a component of transaminase systems (19) and also of ami
acid decarboxylase systems (20). It is also essential to normal tryp
phan metabolism (21, 22). It is possible that pyridoxine deficiency lea
to some defect in apoferritin formation or breakdown which may, in tu
affect iron absorption. On the other hand, further study may yield da
for the elaboration of a new theory of iron absorption.

The increased retention of copper in pyridoxine deficiency is extreme
interesting, though also difficult to explain with the few facts available
present. Whether the copper retention is the result of an effect of py
doxine deficiency *per se* on copper absorption or excretion or whether it
a secondary effect due to changes in iron metabolism cannot be stat
Serum copper has been shown to be slightly lowered in pyridoxine-deficie
swine (23), and whole blood copper in pyridoxine-deficient dogs (2
Since copper is apparently excreted much as other heavy metals throu
the urine and bile (25–27), there is no evidence that copper absorption
regulated by body need to the extent that iron is regulated. Hence,

serum copper might not be expected to exert an influence on copper orption.

3ing *et al.* (28) demonstrated a considerable increase in total copper in ing rats given 0.5 mg. of iron per day intraperitoneally when compared imilar rats given the same amount orally. The iron (total) of these two ups was 6.78 mg. and 2.12 mg., respectively. The copper in the group en iron intraperitoneally was as high as in a group receiving 0.025 mg. copper intraperitoneally per day along with 0.5 mg. of iron orally. This er group had a total iron content of 3.63 mg. This would suggest that per absorption may be influenced by the iron levels in the body. The ervations reported in the present paper and unpublished data from our oratory seem to suggest the same relationship. The converse, *i.e.* that per affects iron retention, has been shown not to be true (29, 30).

t has been suggested that copper enhances the utilization and mobiliza- of iron for hemoglobin synthesis (29, 31, 32). This function, however, s not explain the increase of copper in a condition in which there is inished utilization of iron and hence a decreased need for iron mobiliza- from the stores, unless it represents an overcompensation in an attempt supply iron to a bone marrow which is unable to use it. This might be explanation for the very high serum iron values, *i.e.* an increased mo- zation coupled with an increased absorption.

t is evident from the questions raised by these findings that more work ds to be done on the interrelationships of copper and iron metabolism their influence on erythropoiesis.

SUMMARY

he total body iron and copper have been determined in pyridoxine- cient and in control rats.

3oth iron and copper were significantly increased in the pyridoxine- cient groups.

t is shown that the "mucosal block" theory of iron absorption is not sistent with the findings in pyridoxine deficiency.

Ve are indebted to Merck and Company, Inc., Rahway, New Jersey, the B vitamins used in this work. Cod liver oil and yeast were kindly ished by Mead Johnson and Company, Evansville, Indiana. Vita- E was supplied by Parke, Davis and Company, Detroit, Michigan.

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LETTERS TO THE EDITORS

ON THE OCCURRENCE OF MONOIODOTYROSINE IN THE THYROID GLAND

rs:

Recently, Fink and Fink,[1] applying the method of filter paper partition
romatography to thyroid hydrolysates from rats injected with radioac-
e iodine, reported that one of the many spots observed in their
tographs corresponds exactly with the position occupied by added mono-
otyrosine, and concluded that monoiodotyrosine is a normal component
the gland. But the possibility that the monoiodotyrosine was formed
ring the hydrolysis of the thyroid tissue was not ruled out with certainty.
rthermore, since chemical iodine determinations were not reported, it
s not possible to decide how much monoiodotyrosine was actually
esent.

We wish to present here additional evidence supporting the view that
noiodotyrosine is actually a major component of the thyroid. This evi-
nce was obtained by applying a very sensitive method for chemical iodine
terminations to the usual paper chromatographic procedure. This
abled us to measure the *specific activity* of the iodine in the monoiodoty-
sine and to compare it with similar activities of other iodine fractions.

Rats were injected with suitable doses of I^{131} and their thyroids were re-
ved after periods of 10 minutes, 24 hours, and 48 hours. The glands of
ree or four rats were pooled and homogenized with 1 cc. of cold 10 per
nt trichloroacetic acid, a procedure which serves to separate inorganic
ine from organic iodine in the tissue.[2] The insoluble residue containing
the organic iodine was separated by centrifugation, washed with 2 per
nt trichloroacetic acid, and hydrolyzed on the steam bath for 16 hours
th 1 cc. of 2 N NaOH. The hydrolysate was filtered through a small fil-
stick, and small aliquots of the clear filtrate were mounted on filter
per.[3] Two-dimensional chromatograms were prepared by the ascend-
technique, butanol-acetic acid-water being used as the first solvent and
llidine-water as the second solvent. Radioautographs were prepared in
der to show the position of the radioactive substances. Two of these
re shown to correspond exactly with the positions taken up by added

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diiodo-DL-tyrosine and monoiodo-DL-tyrosine, as determined by sprayin with diazotized sulfanilic acid. The conditions used here were not sui able for location of the thyroxine fraction but were chosen primarily t yield a good separation of the mono- and diiodotyrosine fractions.

Interval after I^{131} injection	Per cent injected I^{131} taken up by thyroid	TCA-soluble iodine		TCA-insoluble iodine hydrolyzed and chromatographed on filter paper							
				Monoiodo-tyrosine		Diiodo-tyrosine		Inorganic iodine		Unidentified spot	
		γ I	Counts per sec. per γ I, × 10^2	γ I	Counts per sec. per γ I, × 10^2	γ I	Counts per sec. per γ I, × 10^2	γ I	Counts per sec. per γ I, × 10^2	γ I	Counts per sec per γ I × 10^2
10 *min*......				2.9	6.5	8.8	3.3				
10 "	0.67	2.4	11.6	3.5	6.0	10.4	3.0	7.1	1.8	2.7	4.4
25 *hrs*.......	24.2	2.3	9.4	6.1	19.2	14.4	19.1	8.4	17.6	5.6	20.0
48 "	8.8	3.3	7.5	5.7	8.8	10.4	11.8	9.5	7.9	4.0	9.2

The table presents our data on the specific activities of several differen iodine fractions prepared with thyroids of rats that had been injecte with I^{131}. It is clear that 10 minutes after the I^{131} injection the monoiodo tyrosine iodine has a higher specific activity than the diiodotyrosine io dine; this finding indicates that the monoiodotyrosine could not hav arisen from diiodotyrosine during the hydrolysis. In 24 to 48 hours, th specific activity of the monoiodotyrosine iodine drops below that of th diiodotyrosine. These findings are well in accord with the view that th former is a precursor of the latter.

It should also be noted (see the table) that monoiodotyrosine forms a appreciable fraction of the total iodine in the hydrolysate. At least 1 per cent of the iodine is present in this form. The inorganic iodine whic appears in the hydrolysate most likely arises from the breakdown of o ganic iodine.

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OME CONDITIONS WHICH AFFECT THE ASSAY OF VITAMIN B_{12} WITH LACTOBACILLUS LACTIS DORNER

irs:

Using a medium containing acid-hydrolyzed casein and tomato juice for e assay of the antipernicious anemia factor,[1, 2] we found the responses *Lactobacillus lactis* Dorner (ATCC 8000) to be very irregular. Skeggs *al.*[3] have cited difficulties with this organism. Use of a tomato juice-east extract-skim milk medium[4] for stock cultures instead of yeast ex-act-glucose agar improved the results, but it was not until further study d to an appreciation of the rôle of aerobiosis that a dependable assay for tamin B_{12} was attained.[5]

Liver extract solids* injectable, per 10 ml. basal medium	Per cent transmission†			
	Autoclaved 15 min.		Autoclaved 3 min.	
	18 × 150 mm.	23 × 150 mm.	18 × 150 mm.	23 × 150 mm.
mg.				
0	44.5	42.0	83.0	97.5
0.001	45.0	41.5	63.0	91.5
0.002	45.0	38.5	69.0	85.5
0.004	45.0	35.5	46.0	75.0
0.010	45.0	39.5	39.0	59.0
0.020	47.0	46.5	40.0	48.5

Medium, 10 ml. of full strength medium contain 0.05 gm. of acid-hydrolyzed sein,‡ 2.0 mg. of L-cystine, 1.0 mg. of DL-tryptophan, 100 mg. of glucose, 60 mg. anhydrous sodium acetate, 0.5 ml. of filtered tomato juice, 0.1 mg. each of ade-ne, guanine, and uracil, 0.05 ml. each of Salts A and B,‡ 10 γ of niacin, 4 γ of pyri-xamine dihydrochloride, 2 γ each of riboflavin, thiamine hydrochloride, and cal-um pantothenate, 1 γ of *p*-aminobenzoic acid, 0.02 γ of folic acid, 0.02 γ of biotin, d NaOH to pH 6.8. *Inoculum*, 20 to 24 hour culture grown in the basal medium us 0.2 mg. of liver extract* centrifuged and resuspended in sterile saline. 1 op of a 1:50 dilution is added per tube. *Sterilization*, autoclaved 3 minutes at 0°. *Incubation*, 37° for 40 hours for turbidimetric determination.

* This extract contains 0.06 γ of vitamin B_{12} per mg. of solids.

† Average of two tubes at each level.

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We have found that maximal growth of *L. lactis* occurred without a vitamin B_{12} *in vacuo* or with the addition of cysteine or sodium thi colate. The effect of conditions influencing the degree of aerobios broth cultures is further indicated by data derived from the use of t of different diameters and of different autoclaving periods. Whe ml. of medium were autoclaved for 15 minutes, maximal growth occu without the addition of vitamin B_{12} sometimes in 23 × 150 mm. tubes always in 18 × 150 mm. tubes. Using 3 minute autoclaving in 2 150 mm. tubes, we have obtained consistent assays for vitamin B various liver and fermentation preparations comparable to results tained with *L. leichmannii* (ATCC 4797) by a procedure similar to published.[3] Shive *et al.*[6] have reported, among other conditions, effects of enzymatic casein hydrolysate and ascorbic acid on the growt *L. lactis*. An enzymatic casein digest would not completely rep tomato juice in our medium.

In confirmation of the report of Shorb[2] we found almost c plete growth suppression of vitamin B_{12} activity (levels of 0.5 to 40 mμgm tube) by folic acid at 5.0 γ per tube and detectable inhibition at 0. Aminopterin was almost completely inhibitive at 0.5 γ, whereas teropt and N^{10}-methylpteroic acid were not antagonistic at 5.0 γ.[7] These eff were common to both *L. lactis* and *L. leichmannii*.

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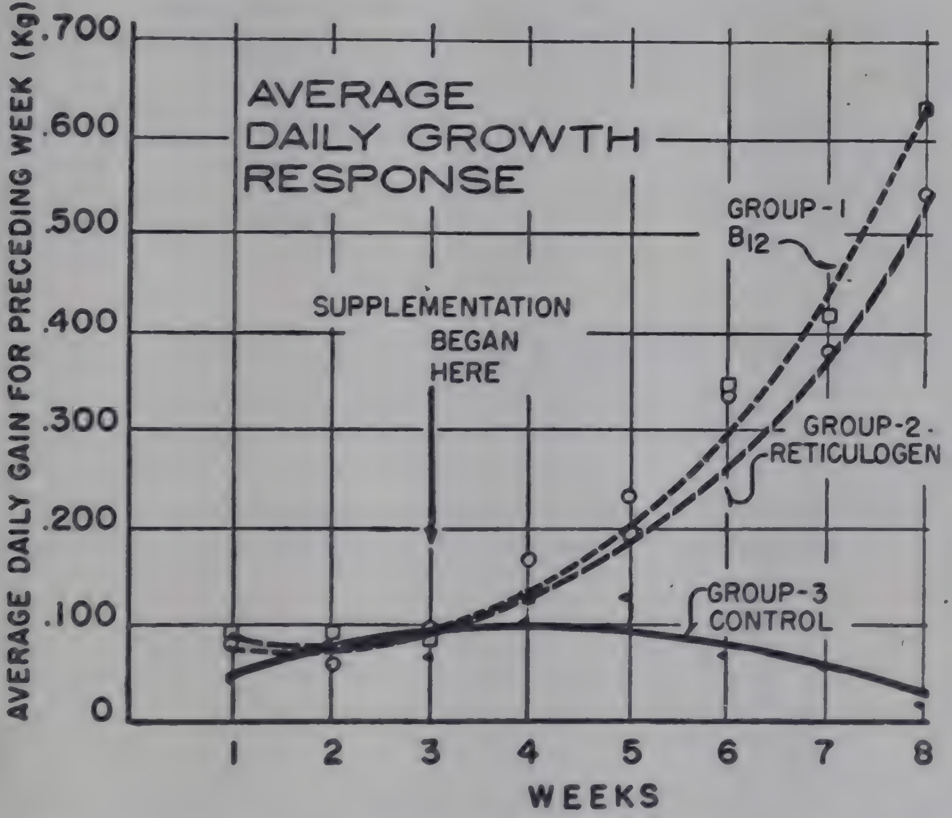
[7] We are indebted to Dr. E. L. R. Stokstad of the Lederle Laboratories for t compounds.

RYSTALLINE VITAMIN B_{12} COMPARED TO ANTIPERNICIOUS ANEMIA LIVER EXTRACT FOR PIG GROWTH

irs:

Crystalline vitamin B_{12}[1] promotes a growth response when injected into aby pigs fed a "synthetic milk" diet containing an isolated soy bean proein[2] as the nitrogen source. The response of baby pigs on this diet to ntipernicious anemia liver extract[3] has been reported previously.[4] In ne present experiment thirteen 2 day-old baby pigs were depleted on the ame diet for twenty-one days and were then divided into three groups. n Group 1, four pigs received by injection 2 γ of crystalline vitamin B_{12}[5] er day. In Group 2, four pigs received by injection 0.1 ml. per day of ticulogen,[3] which assayed 19 γ per ml. of vitamin B_{12} by the *L. leich-*



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[5] Generously supplied by Merck and Company, Inc., Rahway, New Jersey, through e courtesy of Dr. D. F. Green.

mannii procedure of Skeggs *et al.*[6] Two pigs died during the depletio period, leaving three to continue on the basal ration. The figure show the average growth responses of the pigs in the three groups.

The graphs show no difference between the response to crystalline vitamin B_{12} at 2 γ per day and the response to reticulogen at 0.1 ml. per day This level of reticulogen given intravenously should be maximal, since th previous work[4] had shown a maximal growth response to the oral administration of 0.25 ml. per day.

The equality of growth response of baby pigs to crystalline vitami B_{12} and to antipernicious anemia liver extract is similar to the chick dat reported by Ott *et al.*[7] and by Lillie *et al.*,[8] and indicates that vitami B_{12} is the limiting deficiency in our basal diet and is the factor which i being supplied by the antipernicious anemia liver extract.

Three pigs died on the deficient diet. All these pigs showed definit gross symptoms, including marked loss of appetite, hind quarter incoordination, unsteadiness, and a tendency to roll over onto the side or back.

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[6] Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem* **176**, 1459 (1948).

[7] Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, **174**, 1047 (1948).

[8] Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, **176**, 1477 (1948).

HE INFLUENCE OF INSULIN AND ADRENAL CORTICAL COMPOUNDS ON THE METABOLISM OF RADIOACTIVE C^{14}-GLUCOSE IN THE ISOLATED RAT DIAPHRAGM*

irs:

Gemmill[1] first showed that insulin can give a striking increase in deposi-
on of glycogen from glucose in the isolated rat diaphragm. This has
een amply confirmed. The diaphragm effect is the only clear cut in-
lin action of any magnitude which has been verified in an isolated mam-
alian tissue. Recently it was reported[2] that in the diaphragm desoxy-

Assays performed in the usual Warburg equipment; approximately 300 mg. of
aphragm per vessel; sections from each diaphragm divided among all the vessels;
ssue from 100 to 130 gm. of female albino rats fasted 24 hours. 2 hour incubations
ıder O_2 at 38° with shaking at 130 cycles per minute; Ringer's phosphate suspend-
g medium, total volume 4.0 ml. Glucose 0.02 M final, insulin 0.1 units per ml.,
rticosteroids 10 γ per ml. C^{14}-glucose prepared from $C^{14}O_2$ by photosynthesis
the tobacco leaf. Zn insulin crystals supplied by Armour and Company, desoxy-
rticosterone by the Schering Corporation. All the figures in the table are in
rms of 100 mg. of wet weight of diaphragm. The values in parentheses are ab-
lute; the others under analytical are relative, with the control as 100.

Experiment	Analytical values			Total relative radioactivity*		
	Oxygen used†	Glucose used	Glycogen formed	Used (glucose)	Found in CO_2	Found in glycogen
	(0.30 ml.)	(0.97 mg.)	(0.29 mg.)			
ontrol	100	100	100	300	20	40
ısulin	93	145	242	430	25	113
orticosterone	97	102	103	300	20	36
esoxycorticos- terone	97	106	58	320	21	22
orticosterone- insulin‡	90	142	144	420	20	77
esoxycorticos- terone-insulin‡	90	140	117	420	21	51

* Counts of CO_2 as $BaCO_3$, and glycogen and glucose as such, measured at densi-
es of 1.7 to 2.8 mg. per sq. cm., corrected to infinite thickness, specific activities,
ıd relative total counts (Armstrong, W. D., and Schubert, J., *Anal. Chem.*, **20**,
0 (1948)).

† R.Q. approximately 0.90 in all experiments.

‡ Both corticosterone and desoxycorticosterone were used as the free sterols,
ot as the acetate ester.

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* Aided by a grant from the United States Public Health Service.

[1] Gemmill, C. L., *Bull. Johns Hopkins Hosp.*, **66**, 232 (1940); **68**, 329 (1941).

[2] Verzar, F., and Wenner, V., *Biochem. J.*, **42**, 35, 48 (1948).

corticosterone, corticosterone, and certain related compounds may preven the usual accumulation of glycogen from glucose and also antagonize th insulin-enhancing effect.

We have followed the distribution of radioactive C^{14}-glucose in the isolated diaphragm of normal rats under the influence of insulin, desoxycorticosterone, and corticosterone. The results of a typical experimen are summarized in the table. A 2- to 3-fold increase in the radioactivit of the glycogen is obtained with insulin, mirroring the increase of glycoge found by analysis. This shows that the insulin accelerates the norma metabolic pathway from glucose to glycogen rather than stimulating glycogenesis from other sources. We have found with insulin a sma but consistent increase in the radioactivity of the CO_2, showing an influence of the hormone on the over-all combustion of glucose.

Desoxycorticosterone and corticosterone, to a lesser extent, inhibite glycogen deposition in confirmation of the results of Verzar and Wenner With both substances the radioactivity of the glycogen was decreased t about the same degree as the drop in the glycogen-formed values. Th mechanism could involve an inhibition of glycogen synthesis or an acceleration of glycogenolysis. If the latter is true, the compounds at leas are not stimulating total combustion, as evidenced by the lack of increased activity in the CO_2 fraction. It is noteworthy that cortical-glycogen antagonism took place without appreciably disturbing the respiration or glucose utilization rates.

It can be seen that under our experimental conditions only a fairl small percentage of the utilized glucose appears as glycogen and CO_2 To what extent the lost fraction is glucose itself in the tissue or carbohydrate intermediates has not yet been determined. No trace of activit appeared in the fat fraction (fatty acid plus non-saponifiable ether solubles) under any of the experimental conditions employed.

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QUINOLINIC ACID EXCRETION BY THE RAT RECEIVING TRYPTOPHAN

irs:

The administration of large amounts of tryptophan to rats results in the xcretion of nicotinic acid and related compounds in the urine.[1-3] One ich product was reported by Singal *et al.*[2] to have nicotinic acid activity or *Lactobacillus arabinosus* when the urine was autoclaved for 15 inutes at 15 pounds pressure with an equal volume of 2 N hydrochloric cid. They reported the preparation of concentrates of the acid-labile ompound and suggested that it was a precursor of nicotinic acid. Early n the investigations reported here the interesting observation was made hat autoclaving urine with glacial acetic acid for less than 1 hour was as ffective in producing nicotinic acid activity as prolonged autoclaving ith 1 N hydrochloric acid or concentrated acid or alkali. This behavior uggested that a non-hydrolytic change was involved and led to the conlusion, supported by the data presented below, that the compound was uinolinic acid (pyridine-2,3-dicarboxylic acid).

Quinolinic acid was isolated from the urine of rats consuming a nicoinic acid-deficient ration similar to that employed by Hankes *et al.*,[4] but ontaining 2.5 per cent of DL-tryptophan. The activity was followed uring fractionation by chemical and microbiological determinations of he nicotinic acid after autoclaving with acetic acid. The isolation proedure involved (1) adsorption on norit from acid solution, (2) elution ith 0.1 N ammonium hydroxide, (3) extraction of the acid with methnol, (4) chromatographing on activated alumina with dilute ammonium ydroxide as a developing solvent to obtain the ammonium salt, and (5) reatment with Amberlite IR-100 H to give the free acid. The latter as then crystallized from 40 per cent acetic acid.

The isolated compound had the theoretical nicotinic acid activity folowing decarboxylation, as measured by microbiological and chemical ethods. The rates of decarboxylation on autoclaving with 1 N hydrohloric acid and acetic acid were the same as those for synthetic quino-

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[1] Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **163,** 344 (1946).

[2] Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. hem.*, **166,** 573 (1946).

[3] Henderson, L. M., and Hankes, L. V., *Proc. Soc. Exp. Biol. and Med.*, **70,** 26 1949).

[4] Hankes, L. V., Henderson, L. M., Brickson, W. L., and Elvehjem, C. A., *J. Biol. hem.*, **174,** 873 (1948).

linic acid.[5] The isolated compound began to sublime at 166°[6] an changed to a new crystalline form (nicotinic acid) with the evolution o gas at 188–190°. When heated rapidly, it melted completely at 232–237 with sublimation. Authentic quinolinic acid, alone or mixed with th isolated compound, behaved in the same manner. Following treatmen with acetic acid, nicotinic acid was isolated and identified by meltin point and mixed melting point. The dimethyl ester (m.p. 53–54°)[7] wa prepared and melted at 53–55°. The ester prepared from authentic quinolinic acid melted at 53–54°; the mixed melting point was 53–54.5°.

In other experiments[8] it was found that 0.1 to 0.25 mM of quinolinic acid was excreted by rats following the intraperitoneal injection o 1 mM of L-tryptophan, or of 3-hydroxyanthranilic (2-amino-3-hydroxybenzoic) acid, daily in five equal doses. Quinolinic acid has been reporte to lead to N^1-methylnicotinamide excretion[9] and to relieve nicotinic acid tryptophan deficiency in the rat.[8] These findings suggest that quinolinic acid arises from 3-hydroxyanthranilic acid, an intermediate in th conversion of tryptophan to nicotinic acid by *Neurospora*,[10] and probabl by the rat.[11] Oxidative opening of the benzenoid nucleus in the 3,4 position and reclosure through the amino group would account for this transformation.

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[5] Kindly supplied by Dr. H. A. Lardy, University of Wisconsin.

[6] All melting points were determined on the Kofler micro block.

[7] Meyer, H., *Monatsch*, **22**, 577 (1901).

[8] Unpublished data.

[9] Ellinger, P., Fraenkel, G., and Abdel Kader, M. M., *Biochem. J.*, **41**, 559 (1947).

[10] Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **70**, 1847 (1948).

[11] Mitchell, H. K., Nyc, J. F., and Owen, R. D., *J. Biol. Chem.*, **175**, 433 (1948).

ON THE SIGNIFICANCE OF MAGNESIUM ION FOR DESOXYRIBONUCLEASE ACTIVITY[*]

rs:

While studying the action of desoxyribonuclease[1] on calf thymus nucleo- otein and nucleic acid preparations, it was observed that the presence sodium chloride reduced the relative viscosity (η_r) of the solutions. ırther observations showed that magnesium sulfate exerted a more po- nt effect in lowering the initial relative viscosity of the nucleic acid solu- ons. These initial effects make less extensive the subsequent change viscosity on which estimates of enzyme activity are based.

Changes in Relative Viscosity of Sodium Thymonucleate Solutions[*]

ucleic acid used	Initial η_r	η_r at t min.	$\Delta\eta_r$	Added
$7W_7$	3.20	2.61, t = 3.5	0.59	0.2 ml. 0.075 M $MgSO_4$
		2.61, " = 18	0.59	
$7W_7$	3.09	2.18, " = 3	0.91	0.2 " containing 2.5 γ enzyme in 0.075 M $MgSO_4$ + 0.25% gelatin
		1.89, " = 11	1.20	
		1.64, " = 21	1.45	
B-11[†]	4.94	3.82, " = 3.5	1.12	0.2 ml. 0.075 M $MgSO_4$
		3.82, " = 27	1.12	
$7W_7$	3.36	3.05, " = 3	0.31	0.2 " 0.14 " NaCl
		3.05, " = 15	0.31	

[*] 4.8 ml. of a 0.075 per cent solution of sodium desoxyribonucleate in 0.025 M eronal, pH 7.5, were put into an Ostwald viscosimeter and η_r measured. Then 0.2 l. of enzyme or salt solution was added as indicated in the last column, mixed, and rther readings of η_r taken.

[†] We thank Dr. M. McCarty for the gift of this calf thymus nucleic acid prepara- on.

The results presented in the table show that 0.003 M $MgSO_4$ alone can ause a drop in η_r equivalent to 1 unit of activity as defined by McCarty.[1] his author's data on η_r were secured by measurements made *after* Mg^{++} ad been added to the system. Similarly, Fischer *et al.*[2] used a degraded agnesium nucleate as a substrate. If one calculates their values for he Mg^{++} necessary to activate a cruder system than McCarty's, it is

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[*] Aided by a grant from the American Cancer Society to the Department of Pre- entive Medicine, recommended by the Committee on Growth of the National Re- earch Council.

[1] McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).

[2] Fischer, F. G., Böttger, I., and Lehmann-Echternacht, H., *Z. physiol. Chem.*, **1**, 246 (1941).

found that “half activity” was secured at 0.0025 M Mg^{++} and “full
tivity” at 0.012 M Mg^{++}.

Assuming a minimum molecular weight of 4×10^4 for the enzyme,
γ (1 unit) represents 2.5×10^{-12} mole. The substrate nucleic acid (
proximately 5 mg.) equals 5×10^{-9} mole, or, assuming the usual po
nucleotide structure, 2×10^{-8} available phosphoric acid group. T
Mg^{++} present equals 3×10^{-5} equivalent. It can be seen that the Mg
and nucleic acid are much closer in magnitude than are the enzyme a
Mg^{++}. Also, it should be noted that McCarty found 0.003 M Mg^{++} c
timal for *varying* amounts of enzyme while the quantity of nucleic a
was *fixed*. Finally, it should be recalled that Hammarsten's detail
studies[3] showed that Mg^{++} in a concentration of 0.006 N exerted a ma
mum effect on the viscosity and osmotic pressure of sodium thymonucle
solutions.

It is therefore suggested that the function of Mg^{++} or other divale
ions, such as Mn^{++}, in this and other biological systems, in which an i
concentration of this magnitude is needed for activity, is to alter the su
strate so that the enzyme system may function. The application of th
principle to studies of several systems will be reported at a later date.

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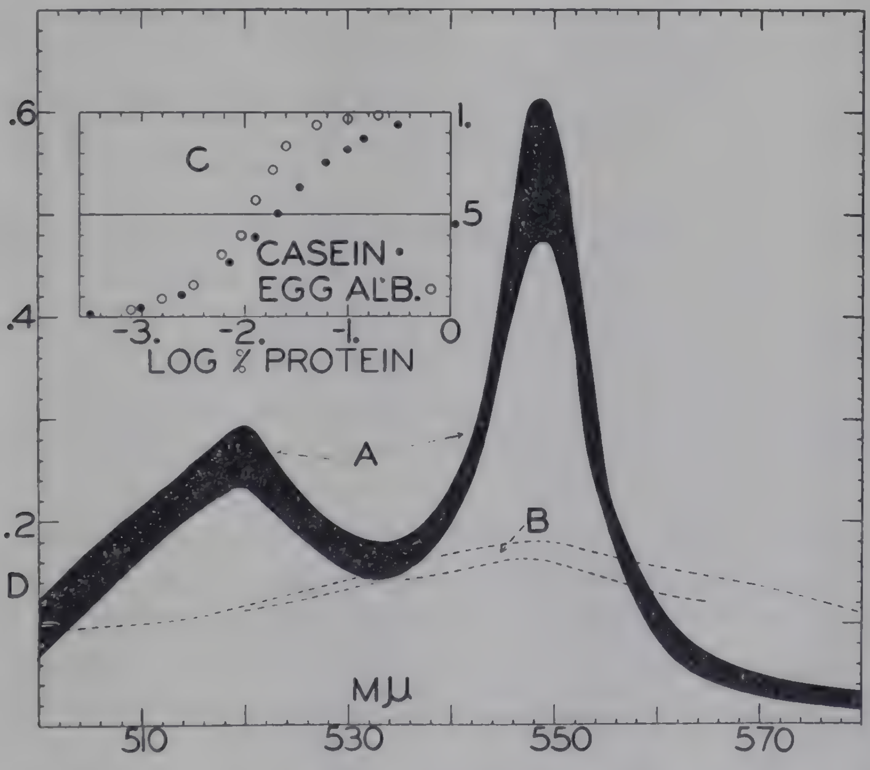
[3] Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).

OORDINATION OF PROTEINS WITH FERROMESOPORPHYRIN

irs:

Lacking space in which to credit all who have studied those so called emochromogens" which involve proteins, we may cite arbitrarily the ob-rvations by Bertin-Sans and Moitessier[1] in 1893 as exploratory antece-ents to the following quantitative relations. Those authors reported e remarkable similarity of absorption spectra given by alkaline solu-ons containing reduced heme and some ill defined proteins, and they nphasized the low concentration at which they detected a protein by eans of such a spectrum.



The similarity of spectra is shown in the figure. Included in the black ea *A* are the optical densities per 1 cm. for the following cases. Each lution contained (or data were reduced to) 0.02 mM ferromesopor-iyrin IX, 0.1 M NaOH, and 0.16 per cent protein. The proteins in-uded were egg albumin (five times recrystallized by Dr. R. M. Herriott), ymotrypsinogen (three times recrystallized by Dr. Herriott), squash obulin (purified by Dr. Vickery), casein (Difco Laboratories), and the iman plasma protein fractions, albumin, γ-globulin fraction II, α_1-glob-in fraction V-1 (from the Department of Physical Chemistry, Harvard

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[1] Bertin-Sans, H., and Moitessier, J., *Compt. rend. Acad.*, **116**, 401 (1893).

Medical School). At a concentration of 1 per cent crystalline zinc ins lin (Eli Lilly and Company) gave a curve falling within area *A*.

Curves similar to those in area *A* are given by pyridine and sever other bases but with slight shifts in the positions of the peaks. The le stable "protoheme" gives similar spectra with proteins but with peak shifted to longer wave-lengths.

Included between the dashed lines *B* are curves for 0.02 mM ferromes porphyrin in 0.1 M NaOH. Turbidity develops so quickly that optic measurements must be made rapidly. Also included is the curve for th same concentration of ferromesoporphyrin with 5×10^{-4} per cent albu min, which enhances dispersion of the solute. The upper curve is tha for a solution to which was added 0.16 per cent pepsin (crystallized b Dr. Herriott).

Bands represented in areas *A* were not revealed by a direct vision spec troscope when *relatively* high concentrations of gelatin (Difco), Bact peptone (Difco), a casein hydrolysate, salmine (Lilly), or any one of sev eral simple substances other than those giving the curves of area *A* wer used.

The inset of the figure (*C*) shows curves of two types relating th logarithms of protein concentrations to degree of transformation calcu lated from optical densities on assumptions similar to those used in th series of papers entitled "Metalloporphyrins."[2]

We shall not draw conclusions until several series of quantitative meas urements shall have been completed. In the meanwhile we shall appre ciate an opportunity to study such proteins, or simpler substances as readers of this Letter may conceive would aid in the elucidation of th phenomena. In view of the low concentration of protein (about 0.0 per cent in the case of egg albumin; see inset of figure) at which 50 per cen change of optical density occurs, none but reasonably pure proteins o known composition will serve if the phenomena are to be referred to pro tein composition.

We are grateful to those mentioned above who have contributed pro teins of very high quality.

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[2] See especially Shack, J., and Clark, W. M., *J. Biol. Chem.*, **171**, 143 (1947).

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